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STUDIES ON HISTOMONIASIS, OR "BLACKHEAD" INFECTION, IN THE CHICKEN AND THE TURKEY.

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I. INTRODUCTION.

UNTIL the sources of blackhead infections were demonstrated and the manner of its transmission became known, this disease doubtless constituted the chief obstacle to turkey rearing in many regions of this country. Fifteen years ago there were relatively few turkeys to be found in New England and one who reared a flock of fifty or one hundred birds was looked upon as unusually successful. At the present time, flocks of several thousand are relatively common and in a recent season one turkey raiser succeeded in rearing nearly twelve thousand turkeys. Mass production has been made possible, first through the recognition and avoidance of the chief sources of infection, demonstrated by laboratory experiment to be mature poultry and contaminated soil, and second by the adoption of the incubator and the brooder house, which not only has made feasible the management of great numbers of poults but has at the same time avoided the more important sources of infection.

Aside from its economic aspect, blackhead infection presents features of unusual scientific interest. Blackhead is a disease brought about by the invasion of the tissues by the protozoön, *Histomonas meleagridis*, first recognized in 1895 by Theobald Smith. Subsequent investigations carried on over a period of many years in the Department of Comparative Pathology at Harvard have served to show that this organism is a flagellate which may be transmitted readily from bird to bird in various ways. In young turkeys, blackhead is progressive in character and therefore almost uniformly fatal. While

serious outbreaks occasionally occur in young chickens, the disease in this species is commonly mild and of brief duration. On recovery from the disease, the protozoön is eliminated from the tissues but may establish itself in the caecal contents in which it continues to multiply for an indefinitely long period. Hence the recovered bird commonly becomes a carrier and its discharges, often containing great numbers of the protozoön, are infective for normal birds. We have demonstrated direct transmission of infection not only by the feeding of caecal discharges containing Histomonas, but also by exposing normal birds both to carriers and to active cases of blackhead, such exposure being comparable to that occurring under natural conditions.

Since Histomonas survives for only a brief period in the material discharged from the caeca, cases of blackhead appearing in young birds that have never been associated with older stock can not be accounted for on the basis of direct transmission. Indeed the origin of many spontaneous cases of blackhead might be even now quite inexplicable were it not for the discovery of Graybill and Smith (1920) that blackhead may be produced by feeding large numbers of the embryonated eggs of *Heterakis gallinae*, the common caecal worm of poultry. While these authors offered the tentative hypothesis that the caecal worms lowered the resistance of the host to the protozoön supposed to be already present, the experimental data which I have collected show quite conclusively that the protozoön is carried by the worm egg. Its microscopic demonstration in the latter constitutes a more difficult problem than might be anticipated and all attempts to this end have been thus far unsuccessful, although Histomonas has been found developing in the intestinal epithelium of a certain proportion of immature caecal worms.

Histomoniasis appears to be unique in respect to its transmission through the agency of the caecal worm. While instances of the parasitization of intestinal worms by protozoa are recorded, no other example is known of an intestinal worm serving as the vector of an infectious disease. The study of Histomonas reveals many features of interest, as for example the phases of its protoplasmic activity, the variation in the appearance of the cytoplasm, the food materials utilized, the wide range in size of individual organisms, as well as inconstancy in respect to the number of flagella present.

From the ease with which experimental infection may be accomplished in incubator-hatched birds which are readily obtained free from all intestinal protozoa, certain problems, as, for example, varia-

tions in virulence and other modifications in biological character, may be undertaken with promise of success. Unlike those protozoan diseases of the human being, which either are not transmissible to lower animals or are not faithfully reproduced in the latter, blackhead lends itself to the study of immunological reactions. Finally, it is possible to make experimental studies of the epidemiology of this infection, in which all essential conditions are adequately controlled.

II. EXPERIMENTAL HISTOMONIASIS IN YOUNG TURKEYS AND CHICKENS, WITH OBSERVATIONS ON VARIOUS FORMS OF TYPHLITIS.

While certain methods of producing blackhead have long been employed by the author as a matter of laboratory routine, much of the resulting experimental data have not been reported. In order to work to advantage in the experimental investigation of this infection, it is essential for most purposes to employ incubator-hatched birds reared under laboratory conditions that will ensure freedom from intestinal protozoa as well as from infectious diseases. By placing birds, on removal from the incubator, in sterilized cages and providing them thereafter with sterilized dishes and sterile food, a stock may be readily kept free of any complicating infection for a period of several months. The success of the regime should be checked from time to time by microscopic examination of the caecal discharges for protozoa and by post mortem examination of any stock birds that may die. Attention also should be given to preventing access of flies or other insects and mice to the cages in which the birds are confined. "Day-old" chickens from certified flocks or "day-old" turkey poults have always been found quite satisfactory. Coccidium infection has appeared on occasion in the latter very early in life, but this is usually transient and need not seriously interfere with experimental work on blackhead.

Infection may be obtained by the introduction of materials containing numbers of living Histomonas into either the tissues or the alimentary tract of young chickens or turkeys. Success will depend in part on the mode of introduction and in part on the infectivity of the material utilized. Histomonas may be found in the following materials which may then be employed as inocula,—liver or caecal lesions in the acute stage from either spontaneous or experimental cases of blackhead, the caecal discharges of laboratory-reared carriers, cultures of Histomonas, and embryonated Heterakis eggs. The

caecal discharges when obtainable in cases of acute blackhead do not always contain Histomonas, but may be employed when this protozoön is microscopically demonstrable. Histomonas is seldom found in caecal cores and the use of the latter for inoculation usually yields only negative results. While the suitability of any diseased tissues for inoculation purposes is ordinarily judged by the number of Histomonas found on examination, this organism has never been observed in the Heterakis egg, and its presence in this case is demonstrated only by trial. Blackhead may be produced also by feeding henyard soil containing numbers of Heterakis eggs to young chickens or turkeys. Certain of the infective materials listed are only suitable for certain methods of inoculation. Thus, aseptically obtained liver lesions serve for any method of inoculation, including parenteral injection. Heterakis ova should be introduced into the alimentary tract, preferably by mouth, in order to hatch, and those materials containing Histomonas in association with bacteria are best introduced into the alimentary tract.

In experimental investigations on blackhead infection, the fact that Histomonas is much more pathogenic for young turkeys than for young chickens must be taken into account. Wide variation in the pathogenicity of this protozoön occurs, a subject which will be discussed in a subsequent section, but even with the more virulent strains the high mortality commonly observed in young turkeys is seldom encountered in young chickens. Thus while recovery from clinical blackhead is extremely rare in turkeys under two months of age, fatal cases in chickens are quite uncommon.

The influence of age on the course of the infection should also be taken into account especially when mature or nearly mature turkeys are utilized. There is a widespread belief that turkeys more than four or five months old never suffer from blackhead, but this is erroneous, since the disease often appears in older birds, which, however, more frequently recover and it is probable that there are also subclinical cases of infection. It is a common experience for turkey raisers to lose considerable numbers of year-old turkey hens from blackhead towards the end of the laying season, but the mortality does not approach that seen in turkey poults. It has also been observed that a large proportion of non-immune adult turkeys survive for many weeks in surroundings that would be promptly fatal to young poults.

Results of Various Methods Employed in the Transmission of Blackhead.

Parenteral inoculation of infective liver lesions.—The production of infection by the injection of Histomonas into the tissues of various species of birds has been reported in an earlier paper (Tyzzer and Fabyan, 1920). Infection has followed subcutaneous, intramuscular, conjunctival, and intravenous inoculation, lesions appearing after an interval of several days at the site of the inoculation, except that lesions may occur in almost any of the internal organs following intravenous inoculation. Following successful breast inoculations in turkeys, secondary lesions regularly appear in the lungs and there is occasionally a further dissemination to other organs, especially the liver. In some species, such as the pigeon, inoculation results in lesions which develop actively for a time but soon regress.

Oral feeding of diseased tissues or caecal discharges and exposure to infected birds.—The experimental production of blackhead through the feeding of liver lesions has been reported in earlier publications (Tyzzer and Fabyan, 1922, Tyzzer and Collier, 1925). This method is rather uncertain, however, even though material is employed that is known to be fully infective when introduced in other ways. Apparently Histomonas may fail to survive the passage of the intestine in numbers sufficient to establish itself in the caeca. Continuous exposure to Histomonas carriers often eventually results in infection. Infection may sometimes also follow the exposure of healthy birds to acutely diseased ones, through the ingestion of food freshly contaminated with infective discharges (Tyzzer and Collier, 1925). Histomonas culture which, when fed orally to one of the series of chickens listed in Table I, failed to produce infection, caused infection when introduced into other chickens by rectal injection. The results of feeding chickens with diseased tissues, caecal discharges, or cultures of Histomonas, or of exposing them to carriers are summarized in Table I. (In this and all following tables, "C" before the bird number signifies young chicken; "T," young turkey; "K," killed; "D," died; and "Typical blackhead," histomoniasis with both caecal and liver lesions.)

Rectal inoculation of infective materials.—As first shown by Browne (1922) and later by Hall and Schillinger (1923), fluid introduced into the cloaca of the chicken is commonly passed into the caeca. Advantage is taken of this physiological response in the production of a type of blackhead infection which corresponds very closely with that which

TABLE I.

INGESTION OF INFECTIVE MATERIAL AND EXPOSURE TO CARRIERS.

BIRD	INOCULATION	RESULT
C 7221	Jan. 26, 1924. Fed suspension of diseased caecum.*	No infection
C 7422	From Apr. 9, 1925, in cage with two chicken carriers.	May 18. *Histomonas* present in discharges.
C 7423		May 15. Numerous *Histomonas* in discharges.
C 7424		May 15. *Histomonas* present in discharges.
C 7425		May 15. Few *Histomonas* in discharges.
C 7426		May 15. Numerous *Histomonas* in discharges.
C 7583	From Dec. 5, 1925, in cage with a chicken carrier.	K. Dec. 16. Typical blackhead.
C 7390	April 9, 1925. Fed caecal discharges of chicken carrier.†	Apr. 13. *Histomonas* in discharges.
C 7391		Apr. 15. *Histomonas* and red blood corpuscles in discharges.
C 8464	Jan. 5, 1932. Fed *Histomonas* culture.‡	No infection
C 8465		No infection
C 8466		No infection
C 8467		No infection
C 8468		No infection
C 8469		No infection

*A control bird rectally injected with a portion of the same material showed Histomonas with blood and mucus in caecal discharges seven days later.

†A portion of this material kept for twenty-three days in 1.5% nitric acid failed to produce infection when fed to five chickens.

‡Other fractions of the same culture material injected rectally on the same date into a control series of six chickens produced infection in all six.

occurs in nature. Indeed rectal inoculation furnishes an easy and rather reliable method of transmitting infection to both turkeys and chickens. A suspension of ground liver lesions may be employed (Tyzzer and Collier, 1925), cultures of Histomonas (Drbohlav, 1924), or the discharges of experimentally infected carriers. Delaplane (1932) also succeeded in infecting young turkeys and chickens reared under the conditions prescribed in our previous papers (Tyzzer and

Fabyan, 1922, and Tyzzer and Collier, 1925) by the rectal injection of diseased tissues and in one case by the rectal injection of Histomonas culture. It is thought that the inoculum is retained rather better in birds that have fasted overnight than in those that are fully fed. The data furnished in Table II will serve to illustrate the results obtained by the rectal injection of young chickens and turkeys with either the caecal discharges of carriers or cultures of Histomonas. While such inoculations are in general successful, it should not be concluded that infection invariably follows the rectal injection of Histomonas. It will be shown elsewhere that failure may result from conditions in the caeca unfavorable to the establishment of Histomonas or from failure to retain the injected material sufficiently long for it to pass into the caeca, as well as from the noninfectivity of materials injected.

Ingestion of Heterakis gallinae eggs.—It was first shown by Graybill and Smith (1920) that blackhead may follow the feeding of embryonated Heterakis eggs to young turkeys. Similar but more adequately controlled experiments carried out by Tyzzer and Fabyan (1920) furnish data indicating that the infective agent is carried within the worm egg. The infectivity of soil from a henyard containing large numbers of Heterakis eggs was also demonstrated by the latter authors in the occurrence of blackhead in thirty-one of thirty-two turkeys exposed, the only failure being in a turkey previously subcutaneously inoculated, and thus probably having some degree of immunity. The results of the experimental exposure of additional series of turkeys to henyard soil under laboratory conditions are furnished in Table III. Thus in a total of forty-nine young turkeys exposed to henyard soil, forty-seven have developed blackhead. The single failure in the series here reported showed no Heterakis. The number of worms is small in several of the turkeys showing typical blackhead, but with the obstruction of the caeca with cores of exudate, a large proportion of the caecal worms are destroyed.

Since the earlier experiments, a considerable amount of additional data has accumulated which furnishes rather convincing evidence of the transmission of blackhead through the agency of the egg of the caecal worm. In the absence of microscopic demonstration, various additional procedures have been resorted to in order to determine whether transmission results from the presence of Histomonas within the worm egg or in associated material. It is customary to collect from chickens large numbers of adult Heterakis which are thoroughly

TABLE II.

RECTAL INJECTION OF INFECTIVE MATERIALS.

BIRD	INOCULATION	RESULT
C 7269	March 15, 1924. Rectally injected with cultures of *Histomonas*.	K. Mar. 23. Typical blackhead.
C 7270		K. Apr. 14. A carrier.
C 7271		K. Mar. 28. Scarring of liver and caeca.
C 7272		K. Apr. 2. Scarring of liver and caeca, a carrier.
C 7273		K. Apr. 4. Scarring of liver and caeca, a carrier.
C 7274		D. Mar. 21. Typical blackhead.
C 7275		Carrier state maintained as late as July 26.
C 7276		K. Mar. 22. Typical blackhead.
C 7281	April 2, 1924. Rectally injected with caecal discharges of a carrier of seventy-six days standing.	K. Apr. 3. Infection. *Histomonas* present.
C 7282		K. Apr. 4. Infection. *Histomonas* numerous.
C 7283		K. Apr. 5. Infection. *Histomonas* numerous.
C 7284		K. Apr. 6. Infection. *Histomonas* numerous.
C 7285		K. Apr. 7. Early typical blackhead.
C 7286		K. Apr. 8. Early typical blackhead.
C 7287		K. Apr. 11. Typical blackhead.
C 7288		K. Apr. 14. Late typical blackhead.
C 7301	April 21, 1924. Rectally injected with *Histomonas* cultures.	K. Apr. 24. Infection. *Histomonas* numerous.
C 7302		K. Apr. 26. Caecal blackhead.
T 7304	May 28, 1924. Rectally injected with *Histomonas* culture.	D. May 30. Infection. *Histomonas* few.
T 7305		D. May 30. Infection. *Histomonas* few.
T 7306		D. May 30. Infection. *Histomonas* few.
T 7307		D. May 30. Infection. *Histomonas* few.
T 7308		D. May 30. Infection. *Histomonas* few.
T 7309		D. May 31. Infection. *Histomonas* few.
T 7310		K. June 4. Infection. *Histomonas* numerous.
T 7311		K. June 11. Typical blackhead.

TABLE II—(*Continued*).

RECTAL INJECTION OF INFECTIVE MATERIALS.

BIRD	INOCULATION	RESULT
C 7313 C 7314 C 7315	March 5, 1924. Rectally injected with *Histomonas* culture.	Carrier state established and continued up to July 28, when all three were employed for testing influence of drugs on *Histomonas*.
T 7317 T 7318	July 28, 1924. Rectally injected with *Histomonas* culture.	D. Aug. 4. Caecal blackhead. D. July 31. Infection. *Histomonas* numerous.
C 7326 C 7328	July 31, 1924. Rectally injected with caecal discharge of carrier.	K. Sept. 8. Infection. *Histomonas* present. K. Sept. 8. Infection. *Histomonas* prseent.
C 7329 C 7330 C 7331 C 7332 C 7333 C 7334	July 31, 1924. Rectally injected with *Histomonas* culture.	K. Sept. 8. Infection. *Histomonas* present. K. Sept. 8. Infection. *Histomonas* present. K. Sept. 8. Infection. *Histomonas* present. K. Sept. 8. Infection. *Histomonas* present. K. Sept. 8. Infection. *Histomonas* present. K. Sept. 8. Infection. *Histomonas* present.
T 7341* T 7342* T 7343† T 7344† T 7345 T 7346	Aug. 23, 1924. Rectally injected with *Histomonas* culture.	Aug. 25. *Histomonas* present, obtained in culture. K. Aug. 30. No gross lesions of blackhead. D. Aug. 30. Caecal blackhead with cores. D. Sept. 19. Chronic, typical blackhead. D. Sept. 2. Typical blackhead. D. Sept. 2. Typical blackhead. K. Aug. 30. Early typical blackhead.

*Naganol injected once prior to, and on two occasions after inoculation.
†Turpentine administered by mouth daily after inoculation,—the other two poults served as untreated controls.

TABLE III.

INGESTION OF HENYARD SOIL.

BIRD	INOCULATION	RESULT	*Heterakis*
T 7515*	Aug. 17, 1925. Henyard soil placed in cage.	K. Aug. 31. Typical blackhead.	42
T 7516*		K. Aug. 31. Typical blackhead.	15
T 7517*		K. Sept. 2. Caecal blackhead.	23
T 7518*		K. Sept. 2. Typical blackhead.	82
T 7519*		K. Sept. 2. Caecal blackhead.	71
T 7520*		K. Aug. 25. Typical blackhead.	30±
T 7521		K. Aug. 25. Typical blackhead.	Numerous
T 7522		K. Aug. 31. Typical blackhead.	21
T 7523†		K. Oct. 1. Healed blackhead. Extensive scarring of liver and caeca.	11
T 7787	Jan. 5, 1927. Soil that had been exposed to freezing and thawing of winter weather without additional contamination for two months preceding, added to food and scattered in cage.	K. Jan. 19. Typical blackhead.	Numerous
T 7788		K. Jan. 20. Typical blackhead.	Moderate numbers
T 7789		K. Jan. 21. Caecal blackhead.	1 (dead)
T 7790		K. Jan. 19. Typical blackhead.	Few
T 7791		K. Jan. 22. Caecal blackhead.	2
T 7792		K. Feb. 1. Caecal blackhead.	Moderate numbers
T 7793		K. Feb. 1. No infection.	0
T 8094	Jan. 8, 1930. Henyard soil placed in cage.	K. Jan 25. Typical blackhead.	101

* "Aviseptone" supplied in drinking water throughout experiment.
† Stovarsol injected intravenously Sept. 10, 11, 12 and 14.

washed and then stored in Petri dishes in a shallow layer of 1.5 per cent nitric acid until the eggs become embryonated. This storage in acid tends to sterilize all material external to the egg shell, and even after only three days' exposure to the acid, worms washed and broken up on various media have failed to furnish any bacterial growth. There is no evidence of any form of Histomonas that is capable of withstanding the direct action of the dilute acid. Other materials such as diseased tissue and discharges containing Histomonas but no Heterakis, after treatment with the acid, have failed to produce infection when fed to normal birds (Table IV). The demonstrable

TABLE IV.

INOCULATION OF DISEASED TISSUES, EXUDATE AND DISCHARGES OF HETERAKIS-FREE CARRIERS AFTER STORAGE IN 1.5 PER CENT NITRIC ACID.

BIRD	INOCULATION	RESULT
T 7132	Sept. 20, 1923. Fed and rectally injected with a mixture of ground liver and caecal lesions and caecal cores from three cases of blackhead, in 1.5 per cent nitric acid for thirteen days.	No infection
T 7133		No infection
T 7134		No infection
T 7138		No infection
T 7139*		No infection
T 7140*		No infection
T 7171	Sept. 15, 1923. Fed and rectally injected with ground caecal cores from two cases of blackhead, in 1.5 per cent nitric acid for five days.	No infection
T 7172		No infection
C 7415	May 2, 14 and 16, June 1, 3 and 9, 1925. Fed caecal discharges of carriers kept in 1.5 per cent nitric acid for 23, 8, 2, 4, 8, 2 and 1 days respectively.	No infection
C 7416		No infection
C 7417		No infection
C 7418		No infection
C 7419		No infection

*Similarly reinoculated with same material on Sept. 21 and 22.

infectivity for normal birds of some of the materials employed in the experiments above tabulated before treatment with acid, does not constitute an adequate control, for there is no question as to the possibility of transmitting infection by appropriate inoculation of non-resistant forms of Histomonas. The object in this case was to obtain evidence as to the presence of resistant forms in materials other than Heterakis eggs.

TABLE V.

INGESTION OF ACID-TREATED *Heterakis* MATERIAL BEFORE AND AFTER EMBRYONATION OF THE EGGS, AND ALSO OF ACID-TREATED *Heterakis* MALES.

BIRD	INOCULATION	RESULT	*Heterakis*
T 7188	Oct. 6 and 8, 1923. *Heterakis* material of Oct. 4 and 5, eggs non-embryonated, fed and rectally injected.	D. Oct. 12. No infection.	0
T 7189		D. Oct. 23. No infection.	0
T 7190		D. Oct. 12. No infection.	0
T 7191		D. Oct. 12. No infection.	0
T 7192		K. Nov. 27. No infection.	0
T 7193	Oct. 20 and 22, 1923. Fed *Heterakis* material of Oct. 4 and 5, eggs embryonated.	D. Nov. 3. Typical blackhead.	Hundreds
T 7194		D. Nov. 6. Typical blackhead.	Hundreds
T 7195		D. Nov. 9. Typical blackhead.	100±
T 7196		D. Nov. 9. Typical blackhead.	Numerous (some dead)
T 7197		D. Nov. 8. Typical blackhead.	150±
C 7395	April 16, 1925. Fed *Heterakis* material of April 13, eggs non-embryonated.	K. Apr. 22. No infection.	0
C 7396		K. May 1. No infection.	0
C 7397		K. May 1. No infection.	0
C 7398		K. May 1. No infection.	0
C 7399		K. May 1. No infection.	0
C 7400		K. May 1. No infection.	0
C 7401		K. May 1. No infection.	0
C 7402		K. May 1. No infection.	0
C 7407	May 1, 1925. Fed *Heterakis* material of April 13, eggs embryonated.	K. May 9. Caecal blackhead.	Numerous
C 7408		K. May 11. No infection	Very numerous
C 7409		K. May 11. Caecal blackhead.	Small numbers
C 7410		K. May 14. No infection.	815
C 7411		K. May 14. Microscopic focus.	7
C 7412		K. May 14. No infection.	589
C 7413		K. May 11. Typical blackhead.	Few
C 7414		K. May 14. Microscopic focus.	66

TABLE V—(*Continued*).

INGESTION OF ACID-TREATED *Heterakis* MATERIAL BEFORE AND AFTER EMBRYONATION OF THE EGGS, AND ALSO OF ACID-TREATED *Heterakis* MALES.

BIRD	INOCULATION	RESULT	*Heterakis*
T 7450	July 1, 1925. Fed *Heterakis* material	No infection.	0
T 7451	of June 26;	No infection.	0
T 7452	July 29, fed *Heterakis* material of July	No infection.	0
T 7453*	25;	No infection.	0
T 7454	Aug. 28, fed *Heterakis* material of Aug.	No infection.	0
T 7455	24, eggs in each instance non-em-	No infection.	0
T 7456	bryonated.	No infection.	0
T 7457		No infection.	0
T 7479	July 13, 1925. Fed *Heterakis* material	D. Sept. 20. Typical blackhead.	200±
T 7480	of June 26;	D. Sept. 20. Typical blackhead.	50±
T 7481	Aug. 6, fed *Heterakis* material of July	D. Sept. 20. Typical blackhead.	100±
T 7482	25;	D. Sept. 21. Typical blackhead.	8
T 7483	Sept. 4, fed *Heterakis* material of Aug.	K. Sept. 21. Typical blackhead.	5
T 7484	24, eggs in each instance embry-	D. Sept. 25. Typical blackhead.	15
T 7485	onated.	K. Nov. 7. Typical blackhead.	25
T 7486	Sept. 18, 1925. Each fed 1/8 of sus-	D. Oct. 5. Typical blackhead.	Very numerous
T 7487	pension of 346 female *Heterakis* of	D. Sept. 24. No gross lesions.	Not examined
T 7488	Aug. 29, eggs embryonated.	D. Oct. 8. Typical blackhead.	70
T 7489	Sept. 18, 1925. Each fed 1/4 of sus-	K. Oct. 26. No infection.	9†
T 7490	pension of 174 male *Heterakis* of	K. Oct. 26. No infection.	2†
T 7491	Aug. 29.	K. Oct. 26. No infection.	0
T 7492		K. Oct. 26. No infection.	0

*Killed before third experimental feeding.

†The birds of this group had been fed non-embryonated *Heterakis* eggs on July 13 and August 6, and possibly reingested eggs that passed through the intestine some of which may have become embryonated on the cage bottom. It is also possible that occasional free eggs adhered to the males and were thus ingested at the September 18 feeding.

TABLE V—(*Continued*).

INGESTION OF ACID-TREATED *Heterakis* MATERIAL BEFORE AND AFTER EMBRYONATION OF THE EGGS, AND ALSO OF ACID-TREATED *Heterakis* MALES.

BIRD	INOCULATION	RESULT	*Heterakis*
T 7486	July 13, 1925. Fed *Heterakis* material of July 9, ova non-embryonated.	No infection, survived.	
T 7487		No infection, survived.	
T 7488		No infection, survived.	
T 7489		No infection, survived.	
T 7490		No infection, survived.	
T 7491		No infection, survived.	
T 7492		No infection, survived.	
T 7493		No infection, survived.	
T 7507	July 20, 1925. Fed *Heterakis* material of July 9, ova embryonated.	D. July 29. No gross lesions.	13
T 7508		D. Aug. 1. Typical blackhead.	Few
T 7509		D. Aug. 4. Typical blackhead.	10±
T 7510		D. Aug. 5. Typical blackhead.	1
T 7511		D. Aug. 5. Typical blackhead.	4
T 7512		D. Aug. 6. Typical blackhead.	4
T 7513		K. Aug. 6. Typical blackhead.	1
T 7514		D. Aug. 7. Typical blackhead.	1

TABLE VI.

SUMMARY OF EXPERIMENTS IN WHICH EMBRYONATED *Heterakis* OVA HAVE BEEN FED TO YOUNG TURKEYS AND CHICKENS.

No. of Birds	Infected	Not Infected
2 Turkeys	2	0
2 "	1	1
5 "	5	0
7 "	7	0
3 "	2	1 (no microscopic exam.)
8 "	0	8
5 "	5	0
8 "	7	1 (no microscopic exam.)
1 "	0	1
1 "	1	0
Total 42 Turkeys	30	12 (two not examined microscopically)
19 Chickens	12	7 (no microscopic exam.)
6 "	6	0
8 "	0	8
8 "	5	3
10 "	6	4
10 "	10	0
4 "	2	2 (no microscopic exam.)
3 "	0	3
1 "	1	0
1 "	1	0
4 "	0	4
4 "	4	0
4 "	0	4
2 "	2	0
2 "	0	2
2 "	0	2
2 "	0	2
2 "	2	0
Total 92 Chickens	51	41

Even more conclusive are the results obtained by feeding samples of Heterakis material, some before and some after the eggs have become embryonated and hence hatchable. Numbers of Heterakis

males from the same source and subjected to the same treatment as the ova-containing females which produce blackhead, also have failed to give rise to infection when ingested by normal birds. The experiments included in Table V illustrate the points in question. These results indicate that infection following the feeding of Heterakis material is dependent on the hatching of the eggs of this worm in the alimentary tract of the bird.

Not all samples of Heterakis ova carry blackhead infection, and in fact a blackhead-free strain of the worm has been propagated in chickens for several generations. It seems probable that only a small proportion of the adult female Heterakis carry infection, as indicated by the no more than occasional invasion by Histomonas of the intestinal epithelium of young worms, and perhaps only a small percentage of the eggs of those are infected. Doubtless the proportion of infective worms will vary widely in various samples of material. Thus, omitting experiments with the strain of Heterakis known to be blackhead-free and those of previously published experiments, embryonated eggs have been fed in ten experiments to forty-two turkeys, and in eighteen experiments to ninety-two chickens, with the results as summarized in Table VI.

Types of Caecal Disease to be Differentiated from Blackhead.

Helminthic typhlitis.—Large numbers of *Heterakis gallinae* developing in the caeca of young chickens produce *per se* a pathological condition which is usually characteristic and readily differentiated from either caecal blackhead or coccidiosis. When Histomonas-free Heterakis material is employed, it is only in rare cases that exudate is produced in such amount as to lead to the confusion of this condition with either of these diseases. Very early or slight infections in either blackhead or coccidiosis may be recognized only by microscopic study of the caecal mucosa.

After the hatching of the Heterakis eggs in the bird's intestine, the young larvae penetrate the epithelium of the caecal glands, a point which apparently escaped the attention of both Graybill (1921) and Uribe (1922) in their studies on the development of this parasite. In stained sections, young worms are found in the epithelium, coiled around the lumina of the glands (Fig. 2). While, at this stage, the head of the young worm may occasionally break through the epithelium, none has been found either immediately beneath the epithelium or in the underlying tissue. There is no appreciable inflammatory

reaction in the vicinity of the young worm. The only demonstrable injury consists of the separation and flattening of the epithelial cells through the pressure of the worm.

In the course of their subsequent development, the worms literally grow out of the glands as noted by Uribe, and eventually come to lie free in the sulci between the folds or elevations of the caecal mucosa. It is at this stage of development that the most pronounced inflammatory reaction has been noted. If the caeca of a young chicken killed from ten to twelve days after ingestion of embryonated Heterakis eggs are laid open, the caecal mucosa often shows a deep red coloration of its numerous elevations due both to local congestion and to the occurrence of punctate hemorrhages (Fig. 1). In this case examination by means of a lens will reveal swarms of young worms lying mostly in the sulci between the elevations of the mucosa. In the more severe cases, there is a small amount of blood-tinged fluid on the surface of the mucosa or the latter is covered with a delicate membrane of exudate.

A study of stained sections shows that, in glands occupied by worms, the epithelium is furrowed by the sharp-edged, lateral flanges of these parasites (Fig. 3). As the worm becomes too large for the gland, its anterior extremity often remains inserted in the gland (Fig. 4) and occasionally, partially grown larvae pierce the epithelium and are found extending through the mucosa without special relation to the glands. This deep penetration appears to take place more frequently when the mucosa is diseased, especially when infiltrated with Histomonas. In one case, a chicken Histomonas-carrier, killed ten days after being fed large numbers of embryonated Heterakis eggs, had the blind end of the caeca filled with exudate which extended also in a layer over the greater part of the remaining mucosa. In this case, Histomonas could not be demonstrated either in the exudate or the caecal mucosa, but was present in the intestinal epithelium of many of the young worms. In the presence of large numbers of growing worms, the epithelium in the depths of the sulci may be not only damaged but destroyed (Fig. 5), while fibrinous or hemorrhagic exudate accumulates in considerable amount. There is also extensive cellular infiltration of the tissues in the neighborhood of the defects.

Thus a pathological condition occurs in the caeca of chickens at a certain stage in the development of Heterakis. Apparently, under certain circumstances, the presence of numbers of the worms may excite a more marked response on the part of the tissues, with an

excess of exudate. Owing to lack of appropriate material, it is not known to what extent the caeca of young turkeys are damaged through the development of Heterakis larvae.

Nonspecific typhlitis of newly hatched chickens and turkeys.—Both chickens and turkeys dying within the first week of life not infrequently show opaque, whitish or yellow-tinged caecal cores, usually of rather firm consistence. It appears important to call attention to this condition, since, without study of stained sections of the caecal wall, it may be confused with blackhead infection.

While the etiology of this form of typhlitis is not wholly understood, it would appear that abnormal conditions arising from functional failure outweigh the inflammatory element in the process and the latter when present may very well represent the reaction of the caeca to a mass of bacteria-containing residuum which cannot be expelled readily. Since fluid is rapidly absorbed in the caeca as shown in observations made in the course of coccidiosis investigations (Tyzzer, Theiler and Jones, 1932), any shortage in the supply of fluid coming to the caeca results in an inspissation of the caecal contents. In cases of typhlitis of the type under discussion, some of the cores are composed in part of fecal material and in part of opaque material of caseous consistence, some cores being firm and tough, others more soft and friable. Such cores are in no instance adherent to the caecal wall.

Stained sections have revealed no more than slight inflammation of the caecal wall and nothing of etiological significance. The epithelium is usually found to be intact. Apparently the cores may be derived from a deposit of non-cellular exudate on a nucleus of inspissated fecal material and mucous secretion. Complete obstruction occurring in the first days of life is often sufficient to cause death. Thus the occurrence of caecal cores in birds dying during the first week of life can not be accepted as evidence of blackhead, and the claim of transmission of the infection through the turkey's egg on the basis of such data should not be accepted. The demonstration of Histomonas and the disease process with which it is associated is necessary for diagnosis.

Caecal coccidiosis.—The lesions of caecal coccidiosis are usually distinguishable from those of other diseases affecting the caeca of the chicken or the turkey, either by the mottled appearance of the caecal wall or by the presence of frank hemorrhage. Even mild cases of this type of infection should be recognized without difficulty on study of stained sections.

Summary

Attention is called to the importance, in the investigation of blackhead, of employing birds reared in the laboratory and kept free of protozoa.

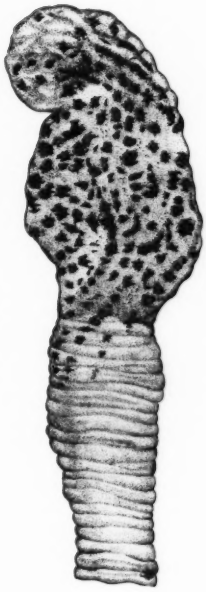
Methods of producing experimental histomoniasis are outlined. The feeding of materials containing Histomonas to young chickens and turkeys may produce blackhead infection but is less reliable as a method than the rectal injection of such materials. Contaminated soil is shown to be an important source of blackhead infection, its infectivity apparently being due to the presence of numbers of embryonated eggs of *Heterakis gallinae*. The ingestion of male Heterakis or non-embryonated Heterakis eggs has not produced blackhead, but the ingestion of the embryonated eggs of this worm is frequently followed by this infection. Thus while the protozoön has not been recognized microscopically within the worm egg, the experimental evidence indicates its presence there, and further that the hatching of the egg is necessary for its release in the bird's intestine. No resistant stage of Histomonas has been recognized and the exposure of discharges and diseased tissues containing Histomonas to 1.5 per cent nitric acid, the reagent utilized for the storage of Heterakis material until the eggs in the latter become embryonated, renders such materials non-infective.

Attention is called to other types of typhlitis occurring in young chickens and turkeys, which are to be differentiated from blackhead infection. The common occurrence in chickens and turkeys of a form of typhlitis of unknown etiology and characterized by the formation of cores obstructing the caeca is reported. This is found at a very early age and should not be confused with blackhead infection. Caecal coccidiosis is readily differentiated from blackhead by the study of the caecal mucosa in stained sections.

Plate I.

Fig. 1. A caecum of a seventeen-day old chicken fed embryonated Heterakis eggs ten days prior to being killed. The caecum has been laid open by longitudinal incision, to show the mucous surface. The elevations of the mucosa of the saccular portion present numerous deep red petechiae (represented in the dark blotches of the drawing) due to minute hemorrhages and congestion. Swarms of small developing Heterakis were found in the depressions of the mucosa, three hundred and twenty-eight being counted from one caecum. × 2.

PLATE I



1

Helminthic typhlitis.—Hemorrhagic petechiae in caecal mucosa of young chicken. X 2.

PLATE II.

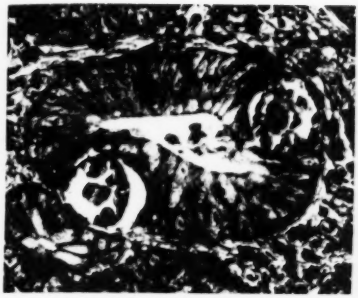
FIG. 2. A caecal gland of a young chicken with a young Hetarakis larva coiled in epithelium and twice sectioned. × 390.

FIG. 3. A somewhat more developed larva, shown in cross section, with body extending free in the lumen of the distended gland. The gland epithelium is furrowed by the lateral cuticular flanges of the worm. From a young chicken eight days after feeding embryonated Heterakis eggs. × 390.

FIG. 4. Heterakis larva, with anterior extremity inserted into a caecal gland, the fundus of which is apparent to the right. From a chicken ten days after the ingestion of Heterakis eggs. × 195.

FIG. 5. Two worms lying deep in a sulcus between elevations of caecal mucosa, shown in cross section. There is local destruction of epithelium, infiltration of adjacent tissue and an outpouring of exudate which in this case filled the blind ends of the caeca. From a chicken ten days after ingestion of Heterakis eggs. × 390.

PLATE II



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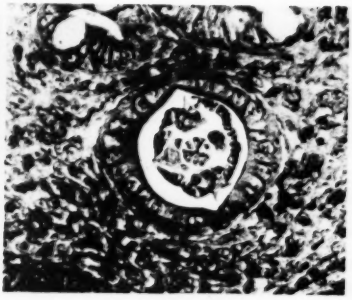
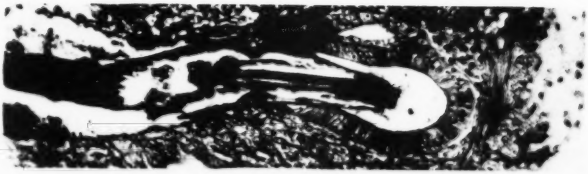


3



4

5

Successive developmental stages of Heterakis larvae in the caecal mucosa. Photomicrographs.

III. *HISTOMONAS MELEAGRIDIS* IN THE AVIAN CARRIER, IN CULTURE AND IN THE CAECAL WORM, *HETERAKIS GALLINAE.*

The morphology of the protozoön of blackhead as found in the tissues of diseased turkeys has already been considered rather fully in papers by Smith (1895 and 1915) and by the author (1919). The tissue-inhabiting form of this organism has the general appearance of a small amoeba, and in fact was classified tentatively by Smith as of the genus *Amoeba.* In seeking to determine the method of multiplication of the protozoön, I found that it presented regularly an extranuclear structure from which division centers originated and that in the course of nuclear division, a long coarse fibril or "paradesmose" was formed. A large proportion of nondividing forms showed intracytoplasmic fibrils suggestive of flagella extending from the extranuclear structures. Further evidence of the flagellate nature of this protozoön was obtained by the study of its movements, at temperatures ranging from 38° to 40° C., in preparations made from liver lesions. It was observed that there was not only a change of shape and a formation of various types of pseudopodia, but also that rhythmic, rotatory movement developed with the appearance of a rudimentary flagellum. Consequently it was obvious that the blackhead protozoön should be reclassified and thus the genus *Histomonas* was created (Tyzzer, 1920). The study of lesions taken at various stages of infection had shown differences in the morphology of the protozoön, *Histomonas meleagridis,* which appeared to have a definite relation to the stage of infection and the reaction of the host tissues. Thus organisms found in the earliest infections or where the tissue was being actively invaded tended to be more drawn out, extending through the interstices of the tissues, and were notably basophilic in their staining affinities; those forms that were found in somewhat older portions of the infected area were large, rounded, closely packed and showed little affinity for stains, their clear hydropic appearance causing them to be readily overlooked; and finally small, dense, rounded forms were found late in the disease process. The last mentioned type of Histomonas shows a somewhat granular cytoplasm which stains intensely, especially with eosin, often occurs in "nests" of from four to twelve or more individuals and is commonly found included within host cells, many of which assume the character of foreign-body giant cells. These small dense, deeply staining forms constitute a much more prominent feature in stained sections of

blackhead lesions than the two types previously described, but structural similarities and the occurrence of transitional forms make it clear that we are dealing with a single species of protozoön.

At this stage of investigation, the exact mode of transmission of the infection was not understood. The small dense forms of Histomonas, especially those that occur within giant cells, commonly show a refractive limiting membrane, and it was suggested that these might represent resistant forms serving to transmit the infection to normal hosts. Support for this view was found in the occurrence of considerable numbers of giant cells containing many such forms, free in the bile ducts of diseased livers. In view of what has since been determined concerning the nature of Histomonas and its transmission, the above hypothesis now appears unlikely and probably erroneous. The dense, limiting membrane appearing in the intracellular forms of Histomonas evidently represents its reaction to the engulfing cell rather than a product of cyclic development and it does not appear probable that the discharge of organisms in the bile plays a significant role in the transmission of the infection.

In considering the question of transmission, the intestinal discharges from acute cases of blackhead in turkeys have been studied. Histomonas is not regularly demonstrable either in caecal discharges or in caecal contents during the active phase of the disease. In fact, on account of the complete caecal obstruction which occurs in severe cases, no caecal discharges are obtainable for long periods, and even when the caeca are not involved throughout, the changes in the caecal contents appear to render this material unfavorable for the maintenance of the organism. Occasionally, however, a thin, blood-tinged discharge may show numbers of large refractive forms of Histomonas similar to those found in the tissues. Obviously, direct transmission of infection from diseased to normal birds may only be expected to take place when the protozoön is present in the discharges.

Flagellated forms of Histomonas were first recognized free in the lumen of the caecum in a number of experimental turkey poults some of which had survived for twenty-two days after inoculation (Tyzzer and Fabyan, 1922). These birds were employed in our first adequately controlled experiment, planned to test out the production of blackhead by feeding embryonated Heterakis ova as reported by Graybill and Smith (1920). The poults used in this experiment were incubator-hatched and rigid precautions were taken to keep them isolated and unexposed to any form of infection other than that intentionally

introduced. Three of these poults when killed twenty-two days after being fed Heterakis eggs showed large numbers of a rounded protozoön exhibiting a type of rhythmic motility quite unlike that of the common caecal flagellates encountered in non-isolated lots of turkeys. Attention should be called to the fact that all three poults in which flagellated organisms were observed, showed no caecal cores although areas of the caecal wall were inflamed and liver lesions were present in each. In a later experiment, similar flagellated forms were noted in a young turkey dying of blackhead twenty-eight days after being fed embryonated Heterakis eggs. The organisms in question were at the time considered to represent the flagellate phase of Histomonas (Tyzzer and Fabyan, 1922).

From this observation, it was natural to look for such flagellated forms in birds that commonly recover from blackhead, i. e. in chickens (Smith and Graybill, 1920), and from the subsequent study of infections in the latter, the carrier condition came to be recognized (Tyzzer, 1924), in which Histomonas may be maintained for an indefinite period through its multiplication in the caecal contents. The possibility of growing the flagellated form of Histomonas from the caecal discharges of the carrier was first demonstrated in this laboratory by Boeck employing the Locke-egg-serum medium utilized in the cultivation of *Endamoeba histolytica* (Boeck and Drbohlav, 1925), and a further study of its growth was continued by Drbohlav (1924). All previous attempts to cultivate this organism from diseased tissue had failed. Some encouragement had been derived from the great numbers of the protozoön sometimes present after twenty-four hours in the culture tube, but these almost invariably had disappeared before the end of the third day. Further attempts to culture Histomonas directly from liver lesions have thus far been unsuccessful. The experimental production of carriers thus paved the way for the propagation of Histomonas in vitro.

Although in the course of subsequent investigations on blackhead, a thorough acquaintance with the more important morphological and biological characters of the flagellated phase of Histomonas was acquired, the publication of an adequate description of such forms has been long delayed. On reporting the chicken as a carrier of Histomonas, the following abstract was published:—"*Histomonas* in its flagellated phase usually shows a single flagellum; motility pulsatile in type, occasionally large forms appear with several flagella. Both uniflagellate and multiflagellate forms show a great variety of amoe-

boid movement, ingest bacteria, cell fragments and small starch granules." (Tyzzer, 1924).

Flagellated forms of Histomonas are figured in Wenyon's Protozoölogy (1926), being here credited through some misunderstanding to Drbohlav, but drawn, as I have subsequently learned, from one of the stained films which I had personally prepared and furnished the latter author.* In Wenyon's discussion of the blackhead protozoön, which is otherwise quite satisfactory, it is unfortunate that a number of inaccuracies should occur such as a typographical error in the spelling of the specific name. Also, the statement that "Tyzzer, 1919 . . . returns to Theobald Smith's original view that the invading organism is actually an amoeba and that the disease is comparable to amoebic dysentery in man" is wholly without foundation and is contrary to my views. I pointed out in this publication that "It is evidently not related to parasitic amoebae of the genus *Entamoeba*," and also that "The presence of an extranuclear body and the character of the nuclear division which is similar to that which occurs in trichomonads suggests strongly that we are dealing with a species of flagellate." Jowett (1911) had long previously "confirmed" Hadley and Amison (1911) in the identification of the blackhead protozoön as a Trichomonas and had pictured aflagellate organisms with extranuclear granules. From the drawings illustrating this author's paper, it appears probable that he observed the extranuclear structure, and in fact in his discussion of the figure he speaks of the organism as a "binucleated" one. Although trichomonads and blastocysts were included as stages of the blackhead organism, the figure in question was described as drawn from a preparation of the caecal wall and illustrates the extranuclear granule in several organisms.

Since the tissue forms of *Histomonas meleagridis* have been described already (Smith, 1895 and 1915, and Tyzzer, 1919), and evidence presented by the latter author of their mode of multiplication through binary division, the present investigation will deal more

* Dr. Wenyon in a personal communication has kindly furnished information as to the labelling of the film utilized for his figures. Since this film bears my personal accession number, it is found that it was prepared from the caecal contents of a laboratory-reared, chicken carrier, fifty-six days old when killed on January 27, 1924. This bird was one of a group infected thirty-five days previously through the feeding of Heterakis ova that had been kept in a 1.5 per cent solution of nitric acid for a period of twenty-two days during which time embryonation had occurred.

especially with the morphological features and biological characteristics of this protozoön outside the tissue of its avian host.

Sources of Material.

The caecal discharges of farm-reared birds are quite unsatisfactory for the study of the flagellate phase of Histomonas. The picture is usually confused by the presence of swarms of intestinal flagellates of various species, and parasitic amoebae are also frequently encountered as well as blastocysts in all stages of development. The most satisfactory material is derived from experimental infections in rigidly isolated, laboratory-reared birds. By the routine procedure described in the previous section which has been followed in this laboratory for a long period of years, large groups of birds are kept free of all parasitic protozoa for periods of several months, in fact as long as the requirements of the experiment demand. Accidental infections may occur, but are then readily recognized and conditions remedied. On the other hand, a sojourn of a very short time under farm conditions is all that is necessary for the acquisition of protozoan infections. Thus *Chilomastix* has been encountered in turkey poults that were kept for a day only in a brooder house that appeared immaculately clean.

Histomonas carriers.—Experimentally infected chickens kept under the regime of isolation already outlined, furnish the most favorable sources of the flagellate forms of Histomonas. In the chicken, the invasion of the tissues is usually of brief duration and with recovery the protozoön quite regularly succeeds in maintaining itself through multiplication in the caecal contents, in this way establishing the carrier state. In young turkeys, on the other hand, the infection is usually fatal within a brief period thus making only the aflagellate, tissue-forms of the protozoön available. However, the loss of pathogenicity of Histomonas in the course of long cultivation, a matter which has been reported briefly in another paper (Tyzzer, 1932), has made possible the use of young turkeys as well as chickens in the present investigation, since the carrier state is as readily established in the former as in the latter birds by the inoculation of an attenuated culture of the protozoön.

As already pointed out, young chickens and turkey poults may be infected with blackhead in various ways under laboratory conditions which exclude other infections as well as the common intestinal protozoa and usually blastocysts also.

(1) The oral feeding of materials containing Histomonas, such as liver lesions taken in the acute stage of the disease, or the caecal discharges of laboratory-infected birds (Tyzzer and Fabyan, 1922, Tyzzer and Collier, 1925), or Histomonas cultures, to laboratory-reared birds has furnished infection but this method is somewhat unreliable. Presumably many of the organisms are destroyed in the passage of the alimentary tract or those which survive may fail to establish themselves in the caeca.

(2) The rectal injection of materials containing Histomonas enumerated in the preceding paragraph furnishes a high percentage of successful results (Tyzzer, 1924, Tyzzer and Collier, 1925). The degree of success obtained varies, but on the whole the method is highly satisfactory. The protozoön is established in the caecum at once, commonly in such numbers that it may be found in the discharges within twenty-four hours and it may persist thereafter for an indefinite period. In two of the chicken carriers of which we have record, Histomonas persisted in the caecal discharges from October thirtieth, the time of inoculation, up to June twelfth, and June twenty-fifth, the respective dates upon which the two birds were killed, i. e. for periods of more than seven months. A daily examination of the caecal discharges also furnishes a valuable indication of what is taking place in the inoculated birds. Such discharges normally consist largely of bacteria and show very few cells. At the onset of inflammatory processes, leucocytes appear and the presence of red blood corpuscles is another indication of injury. If the caecal walls become extensively diseased and exudate accumulates, the caeca become occluded and the discharges cease to appear. With recovery, the mass of exudate is either loosened and discharged, or in some cases digested away, so that function is resumed, as shown by the reappearance of the caecal discharges. Thus by following the changes in the cell content of the caecal discharges of a series of inoculated birds, as well as the number and appearance of Histomonas present from day to day, and by killing some of the birds after appropriate intervals for the study of tissues, it is possible to follow step by step all stages of infection.

(3) Large numbers of mature *Heterakis gallinae*, preferably hundreds if collected from market chickens, may be kept in 1.5 per cent nitric acid in Petri dishes until most of the fertile eggs become embryonated, when they may be fed to young chickens or turkeys (Tyzzer and Fabyan, 1922, Tyzzer, 1926). A small number of worms

from certain birds may suffice. Histomonas seldom appears in the caecal discharges before the eighth day after feeding embryonated Heterakis eggs but isolated organisms have been observed earlier in some instances,—at the end of the sixth day in one case.

Cultures.—Histomonas is usually readily isolated from the caecal discharges of a laboratory-reared carrier in mixed culture with bacteria. Blastocysts when in large numbers in the caecal discharges seriously interfere with the isolation and continued cultivation of this protozoön. Young birds may be kept free of blastocysts by rearing them under the laboratory regime already outlined. The richer media originally used, such as the L. E. S. medium of Boeck and Drbohlav (1925), have been replaced by media which are less favorable to bacterial growth. Cultures on the medium now routinely employed are not only free from the offensive decomposition which occurs in egg media, but have the advantage of being much longer lived. The addition of a small amount of rice starch to the fluid of the culture, as advocated by Dobell and Laidlaw (1926) in the cultivation of *Entamoeba histolytica,* has proved of great value. When ordinary commercial rice flour is employed, it is made available for the protozoön through the action of the bacteria present which break the large starch grains into minute particles. Some of the refined rice starches which come finely ground appear to be quite as favorable for the cultivation of Histomonas as the coarser grades. While one of the strains now under cultivation was first isolated on the L. E. S. medium of Boeck and Drbohlav, the following medium has proved quite satisfactory, not only for the continuous culture of the organism, but also for its isolation from caecal discharges.

Agar 14 grams, sodium chloride 6 grams, and potassium acid phosphate (KH_2PO_4) 2.7 grams are dissolved in 900 c.c. tap water by heating in the autoclave. After allowing the agar to cool to 55° C., the beaten white of one egg diluted with a small amount of water is added, and the mixture is then cooked in the Arnold sterilizer for 45 minutes, being taken out and shaken well every fifteen minutes during the process. After allowing the coagulated egg white to settle, the agar is filtered through cotton, adjusted to pH 7.2, passed through filter paper, tubed, autoclaved and stored until needed. To complete the preparation of the medium, the requisite number of tubes are heated in boiling water, slanted and a small amount (about the volume of a wheat grain) of fine rice starch sterilized by dry heat is added to each tube. Normal saline containing 5 per cent sterile horse serum is then added in sufficient amount to cover the slants. It has been

customary to add also a small amount of dry sterilized animal charcoal to the fluid of each tube, this not being necessary but apparently increasing the longevity of the culture.

The life of the individual culture may be prolonged by adding small amounts of starch at intervals. As the starch disappears, the protozoa become fewer and less healthy, as indicated by their granular and shadowy appearance, but with the renewal of the starch, they regain their bright hyaline appearance and increase in number. The nature of the bacteria associated with Histomonas on its isolation from caecal material is of considerable importance in its subsequent propagation. Some cultures are obtained in which the starch is rapidly destroyed, thus eliminating the food supply of Histomonas. In one isolation which furnished an abundance of protozoa in the first tube inoculated, the increase in the rate of starch destruction in successive transfers rendered it impracticable, if not impossible, to maintain the strain in cultivation. On the other hand, some isolations have furnished optimum conditions for the propagation of Histomonas, the starch on account of the slowness of its destruction being available for a long period. Thus, one strain of Histomonas maintained in culture at present often survives for more than three weeks in the test tube without addition of starch. Intermediate between these extremes are cultures, the transfers of which become extremely rich in Histomonas for a time, but are nevertheless short-lived.

The protozoön usually eventually dies out in the culture tube, even if the supply of starch is kept up, probably from the gradual accumulation of products of bacterial growth. Starvation is thus not the only lethal condition arising in the culture tube. It has been found that the products of degeneration and death of the protozoön itself have no appreciable deleterious effect upon its growth. Thus a culture which for a time is extremely rich in organisms and later as the result of starch starvation shows only a few, degenerated forms may be fully revived by the addition of starch. Histomonas will survive a marked concentration of the medium through the evaporation that occurs during weeks of incubation. Thus the fluid may be reduced by one-half its original volume, or to one-fourth the total medium. The capacity of the organisms for survival in culture when starch and fluid are renewed is illustrated by the following.—A tube inoculated on March 10, 1930 showed large numbers of Histomonas a week later. By maintaining the supply of starch and by adding at intervals water, saline, or saline with serum to offset evaporation, the culture

was maintained in a single tube until January 24, 1931, i. e. for over ten and one-half months. On one occasion a considerable amount of fluid was removed from the tube and replaced by fresh saline with 5 per cent serum, the only material otherwise removed being small amounts of sediment taken at long intervals in order to test the viability and infectivity of the culture. Thus a much greater longevity has been obtained in Histomonas cultures than has been the case with other parasitic protozoa grown in association with bacteria.

It is apparent that the agar, serum-saline, starch medium that is now employed as a matter of routine is not *per se* ideal for the cultivation of Histomonas, but is rendered favorable by the action of bacteria. It has been repeatedly noted that when tubes of this medium are inoculated with a generous amount of sediment rich in Histomonas, it is difficult to demonstrate the protozoön twenty-four hours later. Concomitant with the growth of the bacteria, the sediment shows after a few days great numbers of Histomonas. It is hoped that further studies will furnish more exact information as to the cultural requirements of this organism. The loss of virulence in strains of Histomonas, which has taken place under long cultivation will be discussed in another paper.

The larvae of the caecal worm.—Histomonas has also been found in the gut of the larvae of *Heterakis gallinae*, but thus far it has not been demonstrated elsewhere in this worm although blackhead infection may be experimentally transmitted to chickens and turkeys by feeding them its embryonated eggs.

The Intracaecal Environment of Histomonas

In the caecal contents of the chicken or the turkey, we have a medium subject to sudden and extreme changes. Thus on the functional evacuation of the caeca, most of the Histomonas present are expelled from the host. In the refilling of the caeca with material from the small intestine, there are doubtless sudden influxes of matter quite unlike that previously present. Extreme variation is also found in the water content of the caecal material which may be of the nature of a foamy fluid or firm and dry in character. Histomonas nevertheless maintains itself notwithstanding the vicissitudes encountered in the normal functioning of the caeca and by rapid multiplication makes good the losses which occur through the periodic emptying of these organs. Conditions may arise, however, which may result in the complete eradication of this protozoön. Occasionally following acute

blackhead with complete caecal obstruction, in association with the formation of caecal cores, Histomonas disappears from the host, and the carrier state fails to become established. Furthermore great differences are noted in the course of the infection in carriers. While laboratory-reared chicks usually serve as suitable carriers and Histomonas is maintained for indefinitely long periods at fairly high and constant levels in their caecal discharges, on occasion similarly reared chickens prove to be more or less unfavorable carriers. If, in the latter case, a number are inoculated on a given occasion, the protozoön becomes established in the caeca of some, while others fail to become infected and some of the infections produced are transient in character. The microscopic study of the caecal discharges may show great variation in the numbers of Histomonas not only in individual birds but from day to day in a given individual, while the general average may be low. There is also a marked variation in the appearance of Histomonas as it occurs under the varying conditions of the caecal contents. When flourishing, the organism appears in large numbers and shows vigorous movement, but under unfavorable conditions it occurs in small numbers and may appear faint, granular, and with feeble motility. Even when occurring in considerable numbers, the organism may appear less refractive than on other occasions. Great variation in its size is noted in different birds and on different occasions in the same host.

During a period in which the infection in the groups of carriers under observation was being maintained at a low level, even tending to die out in some individuals, it was observed that a large proportion of Histomonas present were infected with *Sphaerita*, few of the organisms above medium size being free from this parasite. The question thus arose whether *Sphaerita* was of itself destroying Histomonas or whether it was merely an index of other unknown lethal conditions. When under such conditions Histomonas was isolated in culture, *Sphaerita* was not carried over, notwithstanding the fact that such cultures were difficult to maintain on account of the starch-destroying activity of the associated bacteria. Thus even under a standardized laboratory regime, the caecal contents of the chicken may vary to such an extent as to be for periods fully favorable for the maintenance of Histomonas and at other times more or less unfavorable. Since the cultivation of Histomonas in vitro is profoundly influenced by the nature of the associated bacteria, it appears probable that the variation in the flora of the caeca may likewise affect the life of the organism.

Morphological and Biological Characters of *Histomonas meleagridis*

This protozoön living in a widely varying environment presents great morphological variation. In an earlier paper (1919), there were recorded differences in size, contour and staining reaction between invading forms, those at a later stage of the infection and those eventually isolated by tissue cells in initiation of reparative processes. There are likewise notable differences in appearance of the free-living forms which are more or less correlated with the nature of the material in which they are found, although there may be considerable variation in those taken from a given source. It appears that the refractive property of the individual protozoön is closely correlated with the nature of the food material utilized; those living on bacteria are non-refractive in contrast with those living on starch, which appear bright and have sharp contours. Refractivity is also more or less dependent on the general health of the individual organism and those that are feeble and degenerating invariably appear faint and granular. The following description deals with the appearance of the protozoön in life, after fixation and in stained films.

In the caecal discharges, Histomonas meleagridis presents a variety of appearances. When thriving under optimum conditions, it is somewhat refractive, shows both active amoeboid motility and rhythmic rotatory movements so that in the absence of other protozoa it is readily recognized. Large clear refractive forms resembling those found in diseased tissue appear at about the time that invasion of the caecal mucosa is presumably taking place, as indicated by the appearance of leucocytes and red blood corpuscles in the discharges, i. e. in the chicken often from the third to the fifth day of infection. The healthy, active forms occurring in the caecal discharges after the carrier state becomes established appear as rounded bodies exhibiting a jerky, rhythmic oscillatory movement and commonly having a wavy-bordered pseudopodium protruding from some portion of the surface. Motility is less pronounced immediately after the cover-glass preparation of the saline-diluted discharge is made, but becomes well marked in the course of two or three minutes at room temperature. On elevation of temperature, the rhythmic beat becomes correspondingly rapid. Under favorable conditions and with high magnification, the flagellum may be seen stroking in one direction against the surface of the protozoön and causing it to turn, at least at room temperature, not more than one-third or one-fourth

of a full rotation at each stroke. The stroke of the flagellum causes counter clockwise rotation in the great majority of the organisms observed in fresh preparations. In the recovery from each stroke of the flagellum, the body may swing back towards its former position, but some progress in rotation is usually accomplished. The regularity of the rhythm is alone sufficient to distinguish the motility of Histomonas from movement imparted to cells by motile bacteria. On one occasion, four active flagella were distinguished projecting from the surface of a living organism.

The study of freshly made preparations shows an increase in amoeboid activity as the organisms come in contact with surfaces. Low, wave-like pseudopodia are extended into finger or arm-like projections with the terminal portions having a frayed-out appearance due to the formation of delicate processes (Fig. 7). As the organisms spread out in contact with surfaces, they appear much larger than in the rounded state. The contour of the organism changes very rapidly even at room temperature so that it is difficult to obtain camera lucida drawings of motile forms or even to sketch them adequately. Hair-like or rod-like processes are often seen projecting from the more rounded forms, and in some instances large areas of the surface are thickly studded with such structures which are constantly being extended and retracted (Fig. 9).

Cytoplasmic inclusions of the nature of coarse bacilli, occasionally starch granules, and more rarely red blood corpuscles are recognizable in the living organisms. The avian red blood corpuscles are not infrequently ingested when hemorrhage is present. In an experimentally infected turkey which had received injections of large doses of "naganol," a German remedy for trypanosomiasis, many Histomonas were found distended with red blood corpuscles, and in many not showing these cells, the cytoplasm was distinctly tinged a light mahogany color, perhaps due to the presence of some haemoglobin compound resulting from the digestion of the red cells. The cytoplasm, when for the most part of granular appearance due to the presence of inclusions, often shows a clear portion at one end of the organism and this portion shows the greatest amoeboid activity. The position of the flagellum furnishes the only obvious basis for orientation of structure in this species, pseudopodia being extended from any portion of the surface. That there is, however, some degree of polarity, probably associated with differences in the specific gravity within the body of the organism, is shown by the preponderating frequency of

counterclockwise rotation. In those forms with a granular interior, the cytoplasm may be considered to be differentiated into ectoplasm and endoplasm. Many forms occur, however, which are clear throughout.

Histomonas even when present in large numbers in the caecal discharges is frequently inactive and is approximately spherical. Even such forms are recognizable, for they differ from host cells in being evenly distributed throughout the preparation without clumping, and in their uniformly smooth, spherical shape. Furthermore, in such spherical forms, a portion of the cytoplasm is often clear and devoid of granules. On the other hand, the diagnosis of a given cell in the absence of characteristic motility is often quite impossible even after long acquaintance with Histomonas in its various forms.

In culture medium provided with rice starch, Histomonas attains a larger average size than in the caecal discharges, although on occasion large organisms may appear in the latter. Culture forms are also in general much more refractive. A large proportion of these contain particles of starch and bacteria in varying quantity. Bone charcoal, mammalian red blood corpuscles and finely comminuted vegetable material are also ingested when added to the medium. If a culture of Histomonas is examined at intervals, on some occasions few organisms will be found showing flagellate motility, nearly all being actively amoeboid (Fig. 8), while on other occasions the majority will be more nearly spherical and show rhythmic flagellate motility. If watched for a time, many such forms on contact with surfaces are seen to become actively amoeboid, while still showing actively lashing flagella. Protoplasmic processes vary greatly in character. Some organisms similar to ones noted in the caecal discharge spread out into a thin, actively motile sheet with irregularly scalloped edges (Figs. 9 and 41); some show smooth, wave-like projections, while others send out slender, finger-like processes. Occasionally hair-like processes, or long straight bands are extended. In other instances the processes are more or less bent or even spatulate in character (Fig. 9). Perhaps the most characteristic of pseudopodia are the long ones which fray out into numbers of delicate, tapering processes (Figs. 40, 42 and 43) which might readily be missed with other than an oil immersion lens.

Histomonas in its flagellated phase shows great variation in size from minute forms measuring no more than 4.5 microns (Fig. 24), to large ones measuring 25 microns in diameter (Fig. 10), and flattened

forms often measuring considerably more. There is comparatively much less size-variation in a sample from a caecal discharge than is presented in one from a culture, and on the whole the organisms average rather larger in the latter medium. A preponderance of small forms may appear, in some instances in cultures a few days old, possibly the result of rapid multiplication, in others under conditions of starch starvation when the organisms are dying off. Many of the small forms appear insufficient in size to engulf starch particles and doubtless live for a time on other material.

A large proportion of Histomonas propagated in medium supplied with rice starch are intensely iodophilic. They may stain unevenly but the clear, non-granular portion of the cytoplasm is deeply colored. The stained portion appears as a cloud, sometimes of even intensity and in other instances somewhat blotchy. When the preparation is treated with full strength iodine solution (5 per cent potassium iodide in saline solution with iodine added to saturation), the iodophilic material escapes in a large globule, especially from organisms that are extended in amoeboid motion when brought in contact with the iodine. A certain proportion of the organisms are not especially iodophilic and these are invariably devoid of starch. While on occasion forms occurring in caecal material may be iodophilic, usually this peculiarity is lacking, probably due to the paucity of starch. Iodine has also been employed to fix Histomonas with pseudopodia extended and for the study of the flagella. The following method has been found useful. First the surface of a clean slide is marked with paraffin to prevent the flattening or crushing of the organisms through the pressure of the cover glass. A small drop of material rich in organisms is placed on the slide and covered with a cover glass. After an interval of several minutes, the organisms will have extended their pseudopodia, a point which should be checked by microscopic examination, when the iodine solution is added in such a way that it will run in from the edge of the cover glass. The protozoa immediately acted upon by the iodine show the outlines of the most delicate protoplasmic processes as in life, while the flagella also stand out very clearly (Figs. 39, 40, 42, 43, 45 and 46).

Other reagents also serve to fix the protozoön in extended form. With formalin fixation, the iodophilic portion of the cytoplasm remains clear, but with Zenker fixation the organism appears granular throughout (Fig. 44). With the addition of osmic acid, organisms immediately acted on tend to burst and discharge their starch granules,

those acted on more gradually show no staining indicative of the presence of lipoid substances.

Histomonas in stained preparations.—The following description is based on the study of films wet-fixed either in Schaudinn's or in Zenker's fixatives, stained by the Heidenhain hematoxylin method, both with and without counterstains, and dry fixed films stained by the Giemsa method. While flattening and distortion of structure occur with the latter method, these features often work to advantage in the study of minute, closely placed structures. Stained preparations serve further to confirm the great variability of Histomonas as observed in life. Size differences are accentuated through the greater degree of flattening in the larger organisms. The cytoplasm shows a wide variation in its reaction to the Giemsa stain. For descriptive purposes, three types of organisms may be recognized on the basis of staining qualities of the cytoplasm although all three intergrade one with another. It is found that the character of the cytoplasm is profoundly influenced by the nature of the food utilized. Thus the large, vigorous, more refractive organisms, i. e. the starch-feeding forms that show an abundance of iodophilic material, are not stained satisfactorily by the Giemsa method. The contour of the organism is represented by a thick, smooth line and the cytoplasm within remains unstained and shows little in the way of structure aside from such starch particles as may be present. Such forms show so little in stained preparations that they have not been adequately represented in the figures furnished. In contrast, those forms that are feeding on bacteria rather than starch show a clear, deeply blue-stained cytoplasm in which bacteria or other inclusions may be seen (Figs. 18–21, 29–31). A third type is represented by organisms the cytoplasm of which is in general granular rather than homogeneous and which takes a purplish rather than a clear blue stain (Fig. 28). In these there may be a peripheral clear layer. The significance of such forms is not understood although it is possible that they represent organisms in the cytoplasm of which there is storage of nutritive material.

The cytoplasm may be homogeneous throughout showing no hint of differentiation into ectoplasm and endoplasm, or such differentiation may be quite distinct. The latter condition is especially true of the granular forms last described. The differentiation into ectoplasm and endoplasm in this species appears therefore to be a temporary one, correlated with a phase of physiological activity. Such differences relative to the character of the cytoplasm are readily distin-

guished in living organisms and have already been considered. The nucleus in both the carrier and the culture forms of Histomonas is in general relatively small, as already noted in those invading tissue, but is brought out in stained films much more readily than in stained sections. There is no fixed size-ratio relative to the cytoplasmic mass, some organisms of any given size having minute nuclei, others larger ones of relatively coarse structure (Compare Fig. 23 and Fig. 28). Stained preparations indicate a wide variation in the chromatin content. The resting nucleus, as seen in stained films shows granules either fairly evenly distributed or lying against the inner surface of its membrane.

Histomonas may be considered to be normally uniflagellate. Occasional large coarse forms occur with as many as four flagella without indication of division of the single nucleus present (Fig. 28). Biflagellate forms also occur rather frequently (Figs. 31 and 32). The flagellum commonly attains a length slightly greater than the diameter of the organism in its rounded state but is frequently considerably longer, while at other times a large proportion of the organisms may show only short, stubby flagella. The structures near the origin of the flagella vary greatly in appearance in Giemsa preparations. There may be a single, deeply stained granule at the origin of the flagellum from which faint diverging lines extend to the adjacent surface of the nucleus. In some instances, there is a condensation of the cytoplasm around the area traversed by these lines (Fig. 32). In many organisms a second granule is seen internal to the one from which the flagellum arises (Fig. 30). In some instances an ill-defined mass of deeply stained material either obscures or replaces the granules. The flagella in multiflagellate forms arise from such a structure which it has not been possible to resolve into well defined granules, one for each flagellum. It appears quite probable that the granule at the origin of the flagellum is of the nature of a blepharoplast and that the second granule is centriolic in character, but the small size of these structures does not lend itself to assurance of interpretation such as may result from the study of larger and more favorable species.

Multiplication is effected through binary division. Binucleate forms occur in great numbers in some material, but multinucleate forms have not thus far been recognized. Nuclear division is observed in aflagellate forms as well as in others with one, two or four flagella. For purposes of comparison, division forms derived from liver lesions planted in culture medium are figured (Figs. 13–16) together with

one from caecal discharge (Fig. 21) and others from cultures (Figs. 33, 36, 37 and 38). The process is quite similar in organisms from all three sources. In contrast to the wide separation of the daughter nuclei observed at the end of nuclear division in tissue forms, the flagellated forms often show two daughter nuclei lying side by side although in some the two nuclei are widely separated. The spindle or paradesmose of the dividing nucleus is often less conspicuous in the flagellated forms studied in films than in the tissue forms studied in stained sections but otherwise it is of similar appearance in both types. It often appears as a bright, unstained thread of considerable length connecting the division centers (Fig. 36). Distinct chromatin granules, evidently of the nature of chromosomes are seen in the successive stages of nuclear division, but the enumeration of these as well as the determination of other minute features of nuclear division have not been attempted on account of the difficulties and potential inaccuracies involved in interpreting structures so minute. No structures that may be interpreted as parabasal or axostyle have been observed. In fact, cell structures other than those of centriolic or blepharoplastic nature, the associated spindle or paradesmose during division and the nucleus itself, appear to be wholly lacking.

A considerable degree of selectivity in regard to food material is shown by Histomonas. In a stained film, some organisms are found that contain only large bacilli, others very minute bacilli, some are loaded with starch particles, and others show no inclusions whatever. A mixture of various types of inclusions is found, however, in a large proportion of individuals. When red blood corpuscles are added to the medium, a certain proportion of the organisms engulf them and in some instances the entire surface of the latter is studded with adherent red cells. Inert material, such as wood charcoal, is taken up in considerable amount.

Histomonas in the caecal worm.—Histomonas has been found in the epithelium of the gut of *Heterakis gallinae* from cases of acute blackhead produced by feeding the embryonated eggs of this worm and also from carriers which had similarly been fed worm eggs. This phase of development has not been adequately investigated. The scant material available has shown the protozoön confined to the epithelium of the gut of ten-day and twelve-day old worms developing in chickens, and several were found similarly situated in a twenty-one day old worm from a young turkey. While the protozoön as found in the worm shows a very dense granular cytoplasm (Fig. 47), the nucleus

and blepharoplastic structures are essentially similar to those of Histomonas occurring elsewhere. Processes having the appearance of pseudopodia have been observed in fixed material, and a typical division form has been seen. The protozoön in this situation lives upon the coarse globules, presumably lipoid material, stored in the intestinal epithelium of the worm, a diet quite different from that supplied by the culture medium employed or by caecal contents. While Histomonas in avian tissue cannot be regarded as an intracellular parasite, its occupancy of the intestinal epithelium of the worm renders it here essentially intracellular. The occurrence of nests of several organisms in the epithelial cells is probably due to multiplication. The mode by which it is transmitted through the worm egg to new avian hosts remains to be worked out.

Attention has already been called to the lack of encysted forms of Histomonas, and its failure to survive in lesions after death or in discharges outside the body. It dies off rapidly in cultures left at room temperature, an overnight exposure being sufficient in some cases to destroy the organism. It nevertheless withstands freezing for an hour or more in culture and on rapid thawing will immediately resume movement. However, freezing overnight at low temperatures, $-15°$ C., is sufficient to destroy it. Experimental evidence of its occurrence in Heterakis ova has already been presented (Section II). In this situation, it furnishes evidence of great resistance, survives New England winters in the soil, and in this way furnishes a means of transmitting histomoniasis to young birds that have never in any way been associated with older ones.

Discussion

The biological properties of Histomonas should be taken into account in consideration of the claims of the transmission of the protozoön through the avian egg to the newly hatched bird. Schlotthauer and Essex (1931) have reported the occurrence of fatal blackhead in turkey poults within the first week of life. Their account and description of the disease in birds over a fortnight old is quite consistent with blackhead but the nature of the cases in which death occurred during the first week of life is not wholly clear. We have long been familiar with a condition in very young chickens and turkey poults which might readily be mistaken for caecal blackhead. The caeca in such cases are completely occluded, with well developed cores but stained sections fail to reveal either Histomonas or any pronounced inflamma-

tion of the caecal wall. This condition is responsible for a considerable amount of the early mortality in certain lots of birds and is associated with an abnormality of the fecal discharges causing a caking of the feathers around the anal opening. The etiology of the condition is not understood other than that the number of cases may be increased by chilling. On the basis of long experience with this condition and with mild cases of histomoniasis, we would not undertake to make a diagnosis of the latter, especially in crucial cases, on the basis of gross pathology, even though liver as well as caeca showed disease. The demonstration of a single case of histomoniasis, occurring among properly isolated, incubator-hatched birds would furnish an important indication of maternal transmission through egg to offspring, but no such evidence is available either in the report of the above authors or in the large series of turkeys and chickens reared under controlled conditions in our own investigations. Histomoniasis is readily recognized in laboratory stock, and is, except in rare instances, a persistent infection with a high probability of spontaneous transmission to other birds of the same group. No explanation is offered by the above investigators as to the mechanism of maternal transmission, how the protozoön gets into the egg, where it occurs in the egg or how it gets to the alimentary tract of the chick. Nor do they give due consideration to the acuteness of blackhead infection in very young birds. In the course of our investigations we have observed inflammation of the caeca at seventy-two hours and death from typical blackhead with extensive liver lesions within six days after the inoculation of young chickens. Thus, under unusual conditions, histomoniasis acquired after hatching might cause very early death.

The following observations may offer some elucidation of the question although they should not be considered as conclusive in nature. If hanging drop preparations are made from a suspension of culture rich in Histomonas, and kept at 38° C. the protozoön persists in good condition and in undiminished number for at least twenty-four hours. If a preparation is similarly made except that a small amount of fresh egg albumen is added, the protozoa are all killed on incubation overnight, and in similar preparations to which a small amount of fresh yolk is added, the survival time is not much longer. If Histomonas is to survive in the avian egg, it would appear that some form more resistant than those occurring in culture is necessary.

The question of the maternal transmission of blackhead through the egg is one of great importance to turkey raisers. The possibility

of such transmission through the agency of some hypothetical resistant form cannot be denied on the basis of negative evidence although no instance or even indication of it has been encountered either in laboratory or practical experience of many years' standing. Its proof would be furnished by the appearance of histomoniasis in carefully isolated laboratory-reared birds, but in no instance to date has infection occurred under such conditions. Hence the evidence for maternal transmission offered by Schlotthauer and Essex is by no means convincing.

The study of Histomonas as it occurs in the avian carrier and in culture has revealed no encysted or resistant form to account for its persistence in soil. Since blackhead may be regularly produced by the routine procedure of feeding the eggs of *Heterakis gallinae* that have been kept in 1.5 per cent nitric acid until embryonated, and also since blackhead may be produced by the feeding of soil, containing Heterakis eggs, that has been exposed to the freezing and thawing of the winter months, the conclusion has been reached that a resistant stage capable of transmitting infection to susceptible birds is carried by the worm egg. The experimental evidence is consistent with practical experience in that growing turkeys will acquire blackhead from contaminated soil without any direct exposure to poultry or older turkeys.

The question may be raised as to whether the forms of Histomonas here described, some possessing a single flagellum and others multiple flagella, represent two rather than a single species. That the forms in question agree in all characteristics other than in the number of flagella renders this possibility rather improbable. In a protozoön showing such a wide range of variability, occasional precocious multiplication of the flagella previous to nuclear division is not remarkable. Organisms are observed in the process of nuclear division with one, two or four flagella, others with none. Multiflagellate forms are not only encountered in caecal discharges and in freshly isolated cultures, but also in cultures that have been maintained for long periods, one for more than three years. The flagella in both multiflagellate and uniflagellate forms, are similar in appearance and mode of functioning, although often shorter in proportion to the diameter of the organism in the multiflagellate form.

The different morphological types of Histomonas previously described in avian tissues and their correlation with stages of blackhead infection are supplemented in the present study by types found in

various other environments. The starch-ingesting forms of Histomonas, from the great size attained, their unsatisfactory reaction to stains, and the readiness with which they undergo dissolution with certain reagents, would appear to correspond to those in the "vegetative stage" previously described in blackhead lesions. Probably much of the difficulty in recognition of histomoniasis in stained section is based on the nondescript appearance of such forms. Without perfect fixation they appear as scarcely more than spaces in the tissue and the small nucleus is often inconspicuous, even on deep staining. The smaller, dense, rounded forms are much more readily recognized. Quite as great a variation in the morphology of the protozoön has been found in its study in culture and in the discharges of carriers as was previously observed in infected tissues.

Difference in type is perhaps based not wholly on environment, for a considerable degree of selectivity with respect to the ingestion of food material is shown in the organisms of a single culture. The ability of the species to adapt itself to sudden changes and to different environments has already been pointed out. Thus, in culture it thrives upon starch, in the bird's caeca it lives largely upon bacteria, during disease it may live on cell fragments and tissue fluid, and in the gut of the caecal worm it is found ingesting the lipoid globules of the intestinal epithelium. The diet of the protozoön appears to affect the general appearance of its cytoplasm whether in life or in stained preparations.

Although the etiological significance of Histomonas to the specific disease known as "blackhead" has not been seriously questioned since its discovery by Smith in 1895, nevertheless from discussion that has occurred at live-stock conferences, it would appear that there are those who not are fully convinced. To the author the question involves scarcely more than a definition of blackhead as a reaction of the host to the invasion of its tissues by Histomonas. The constant association of Histomonas with the lesions of this disease in whatever organ they may occur, the reaction of the host tissues to the protozoön constituting the disease process, the correlation of repair with the elimination of the protozoön, and the experimental production of typical disease by the inoculation of material containing no organism thus far demonstrable other than Histomonas, appear to constitute convincing evidence of the etiological significance of this protozoön. Thus we regard histomoniasis and blackhead as synonymous terms and believe that the use of the latter name except for the specific disease in question will result only in confusion.

No evidence is available of the presence of a virus other than Histomonas or of any other factor peculiar to the disease. In the next following section it will be shown that the subjection of a culture of Histomonas to sufficiently low temperatures to destroy the protozoön renders it noninfective although the associated bacteria survive such treatment. Whether or not it is possible to satisfy the requirements of Koch's postulates, or whether the organism in question has been successfully propagated in vitro, the multiplication of any protozoön within the tissues of a higher animal may not be disregarded with impunity in the consideration of disease, especially when the reaction to the protozoön appears to constitute the disease. By employing diseased tissues, caecal discharges, cultures, or embryonated eggs of the caecal worm which contain Histomonas, blackhead is produced as a matter of laboratory routine, quite as readily as any of the well known bacterial infections. It is true that in experimental work on blackhead it is important to employ clean, laboratory-reared stock in order to avoid the possibility of encountering adventitious infection, or immunity resulting from previous infection, and also to avoid the difficulties of diagnosis resulting from the presence of swarms of other species of protozoa in the caecal contents. A rabbit or a guinea pig from almost any source providing it is in good health, may meet the requirements of the bacteriologist, but the employment of birds, not laboratory-reared, will for most purposes yield wholly unreliable data in the investigation of blackhead. It is believed that much of the difficulty involved in the experimental work on this subject lies in the failure to recognize this principle and perhaps also in a lack of ability to identify Histomonas on the basis of its morphology. In the latter case the investigator works largely in the dark with reference to the presence of the infective agent in any material that he may employ, and also in the interpretation of his results.

In regard to the systematic position of Histomonas, it is included by Wenyon in the family MONADIDAE Kent, 1880, who calls attention to its resemblance to a member of the genus *Oikomonas*. While the process of nuclear division of Histomonas is in many respects similar to that described in various species of *Trichomonas*, it shows, apart from nucleus and flagellum, none of the structural differentiation of the better known intestinal flagellates and is moreover actively amoeboid. Thus it appears preferable not to undertake anything further in the way of classification until other more or less related species are more carefully studied.

Summary

Routine methods of producing histomoniasis or blackhead that will furnish sources of material for the study of *Histomonas meleagridis* are reviewed.

A method for the isolation of Histomonas from the caecal discharges of carriers and for its continuous propagation on artificial medium in association with bacteria is described.

The lack of satisfactory evidence of maternal transmission of blackhead infection through egg to offspring is pointed out.

The morphology of *Histomonas meleagridis* as it occurs in the caecal discharges is similar to that of the forms that develop in culture, although a somewhat greater average size is attained in the latter. The species may be considered as normally uniflagellate, but there occur multiflagellate forms of large size which may show as many as four flagella. The cytoplasm may be homogeneous, staining a clear blue by the Giemsa method, or it may remain practically unstained in starch-ingesting individuals. There are also forms that show a purplish staining, granular cytoplasm and some of these present a clear ectoplasmic layer. Starch-ingesting organisms are intensely iodophilic, the iodine staining material being concentrated in a portion of the cytoplasm devoid of granules. In the caecal contents, in the presence of hemorrhage, avian red blood corpuscles are frequently ingested and various materials in addition to starch and bacteria are ingested in culture.

The forms here described from caecal discharges and cultures correspond closely with respect to the appearance of the centrioblepharoplastic structures, the nucleus, and the process of nuclear division, with those previously described in diseased tissues. Perhaps the most striking feature of Histomonas is its oscillating movement in which there is partial rotation on the stroke of the flagellum with a tendency to swing back as the latter recovers. The flagellated forms quickly take on amoeboid activity in cover glass preparations, becoming extended especially when in contact with surfaces and at the same time may retain their flagella over a long period. There is great variety in appearance presented in the amoeboid activities of the organism which may show sluggish changes of shape or rapidly extended pseudopodia which may be wave-like, finger-like, in the form of slender bands, rods or hair-like processes, but those that are frayed into fine, tapering, hair-like projections are probably more typical of the species. On occasion, the organism may flatten itself

out into a thin motile sheet of irregular outline. In the intestinal epithelium of Heterakis, Histomonas is found to ingest the lipoid globules of the invaded cells and presents a dense granular-appearing cytoplasm.

No encysted form of Histomonas has been discovered thus far, and, although there is experimental evidence indicating the presence of a resistant stage in the Heterakis egg, it has not been microscopically demonstrated in the latter.

Plate III.

In figures 6 to 9, Histomonas is represented as in life at 1020 diameters.

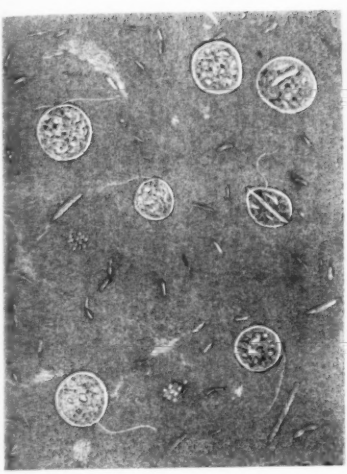
Fig. 6. Histomonas from a freshly passed caecal discharge of a chicken carrier. Such forms show a jerky rotatory movement, are more or less rounded and are commonly not refractive.

Fig. 7. Histomonas from the same material as above but after an interval of five minutes after the preparation was made. The organisms have commenced active amoeboid movement and are spreading out with ragged-edged, sheet-like pseudopodia.

Fig. 8. Amoeboid aflagellate forms of Histomonas from a vigorous six-day culture.

Fig. 9. Selected extreme types from Histomonas cultures. The two lower drawings at the left depict the change in outline of a single organism within an interval of about a minute and a half at room temperature. Below and to the right an organism is shown with surface studded with slender rod-like processes, probably not normal although actively motile. The uppermost organism represented has ingested four mammalian red blood corpuscles from those supplied in culture, somewhat below this is a non-motile clear hyaline sphere, and to the right is a small actively motile hyaline form spread out in a thin sheet.

PLATE III



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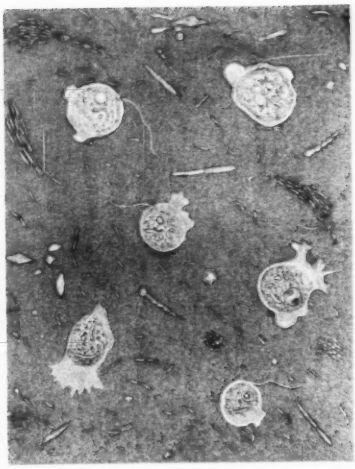

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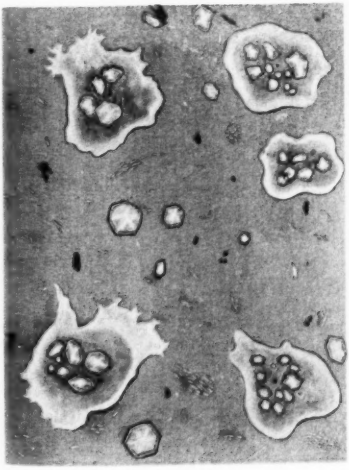

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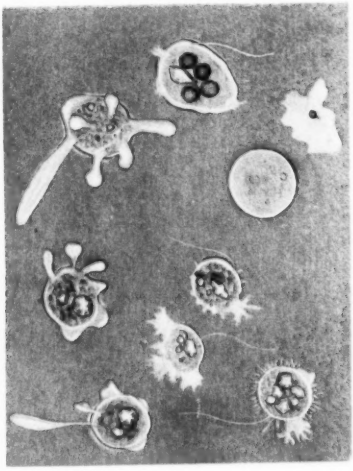

9

Histomonas in caecal discharge (above); various forms seen in culture (below). X 1020.

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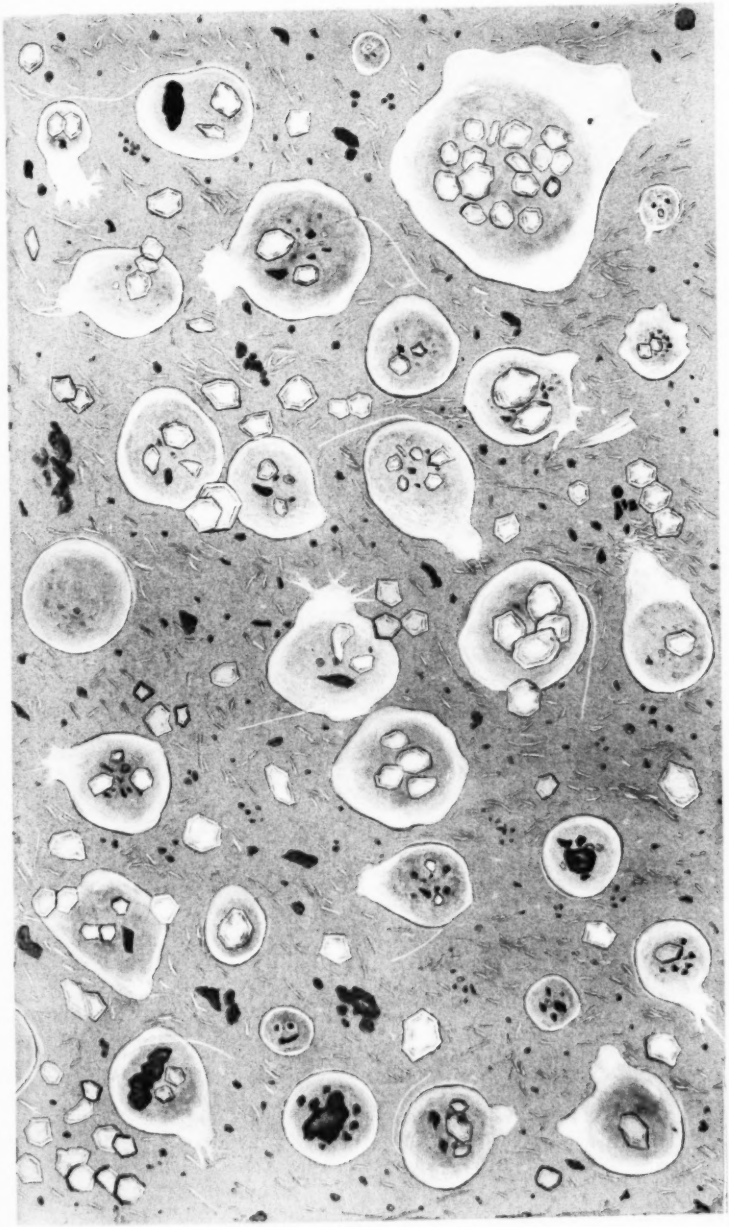
Plate IV.

Fig. 10. Sediment from a rich culture of Histomonas magnified to 1300 diameters. The bright angular bodies both free and within the organisms represent starch particles, the black material "bone black." There are bacilli scattered throughout the field. The flagellum even when present is not always readily distinguished. Such starch feeding forms are in general larger and much more refractive than those usually encountered in the caecal discharges of carriers (Compare with Figs. 6 and 7). The characteristic pseudopodia fraying out into ragged endings are apparent in several of the organisms. In the upper right hand corner are selected large and small forms indicating the extremes in size variation.

PLATE IV



10

Histomonas meleagridis in fresh preparation from culture. X 1300.

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Plate V.

Figures 11 to 21 inclusive are from a thirty-eight day old turkey from the first well controlled experiment in which blackhead infection was produced by feeding Heterakis ova previously treated with 1.5 per cent nitric acid. Death, October 14, 1921, from blackhead at the end of four weeks from the feeding of Heterakis ova. Giemsa stained films. Figures 11 to 17 were from liver-lesion material that had been in culture medium from two to twelve hours; figures 18 to 21 from caecal material in culture medium from five to twelve hours. Figures 25 to 28 are drawn from stained films of caecal discharges of a chicken carrier experimentally infected thirty-five days previously. All other figures are from a Giemsa stained film of a Histomonas culture. All figures reproduced at 1150 diameters.

Fig. 11. Large, lightly stained Histomonas showing an intracytoplasmic filament extending from blepharoplast,—from liver lesion twelve hours in culture medium.

Fig. 12. Deeply stained granular organism showing a similar filament,—liver lesion twenty-four hours in culture.

Fig. 13. Division form, the daughter nuclei widely separated on paradesmose,—same preparation as Fig. 12.

Figs. 14 and 15. Large forms in process of nuclear division, possibly some disarrangement of structure through pressure in preparation of film,—same preparation as Fig. 12.

Fig. 16. Binucleate organism with double extranuclear granules clearly shown,—same preparation as Fig. 12.

Fig. 17. Histomonas showing a short flagellum as well as an internal filament,—from liver lesion two hours in culture medium.

Fig. 18. Histomonas with short flagellum, derived from caecal material that had been twelve hours in culture medium. The cytoplasm shows ingested bacteria.

Figs. 19 and 20. Similar forms from caecal material five hours in culture medium.

Fig. 21. Division form showing two flagella derived from caecal material five hours in culture medium.

Fig. 22. Starch laden Histomonas, no flagellum visible. Culture, Giemsa.

Fig. 23. Form illustrating small size of nucleus in many organisms, cytoplasm homogeneous and intensely basophilic. Culture, Giemsa.

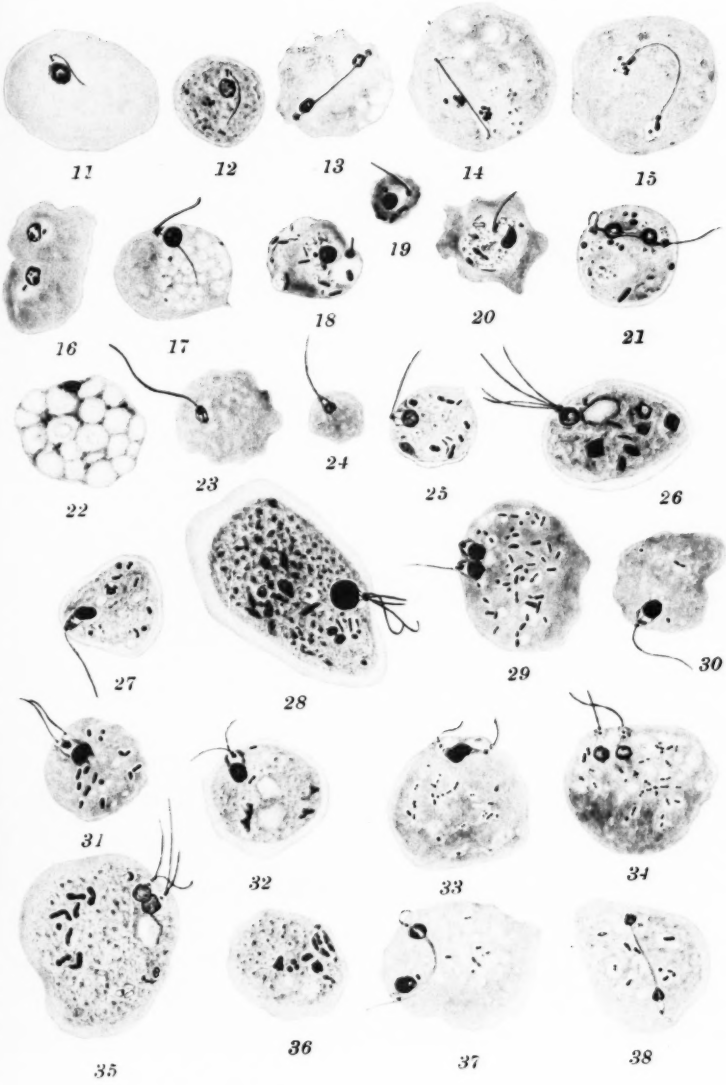
Fig. 24. Minute flagellated Histomonas, cytoplasm basophilic. Culture, Giemsa.

Fig. 25. Uniflagellate form with ingested bacteria. Caecal discharge, Heidenhain's haematoxylin.

Fig. 26. Uninucleate, quadriflagellate Histomonas with starch grains and several bacteria. Caecal discharge, Heidenhain's haematoxylin.

Fig. 27. Uniflagellate form. Caecal discharge, Giemsa.

PLATE V



Histomonas from stained films. X 1150.

FIG. 28. Giant, quadriflagellate form with large nucleus. The flagella arise from a single blepharoplastic mass. Cytoplasm differentiated, with clear ectoplasm, and a coarsely granular endoplasm containing bits of material, possibly starch. Caecal discharge, Heidenhain's haematoxylin.

FIG. 29. Binucleate form with a single flagellum visible. Culture, Giemsa.

FIG. 30. Uniflagellate form, two granules near origin of flagellum.

FIG. 31. Biflagellate, uninucleate form. There is a granule at the base of each flagellum, and a single coarser mass below.

FIG. 32. Biflagellate, uninucleate Histomonas, with a zone of dense cytoplasm surrounding blepharoplastic area.

FIG. 33. Division form, a short flagellum arising from each blepharoplast.

FIG. 34. Biflagellate, binucleate organism with blepharoplastic granules distinct from centrioles.

FIG. 35. Quadriflagellate, binucleate form. Nuclei in close apposition.

FIG. 36. Aflagellate culture form in early division. Chromosomes and paradesmose visible.

FIG. 37. Division form with two short flagella, nuclei widely separated on paradesmose.

FIG. 38. Division form showing no flagella.

Plate VI.

The organisms are represented in this plate at 1150 diameters.

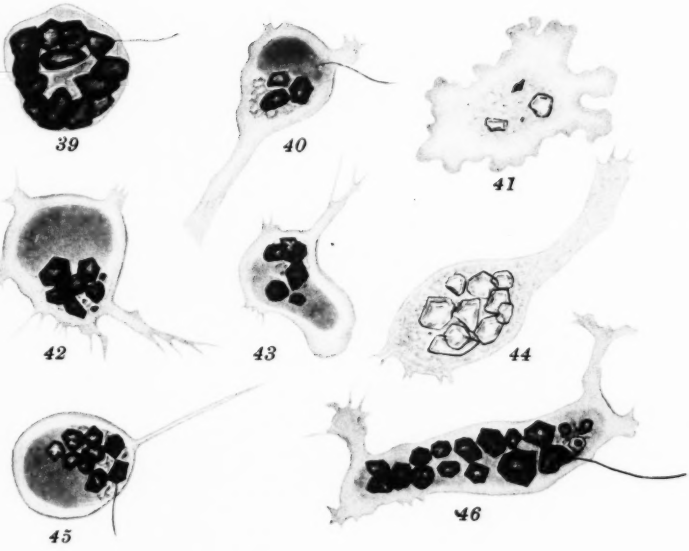
Figs. 39, 40, 42, 43, 45 and 46. Histomonas from culture, killed and stained with strong iodine solution after being allowed time in cover glass preparations to become actively amoeboid. The included starch grains are stained intensely by the iodine and the diffuse cloud of iodophilic material is represented by deep shading. The organism shown in the first figure has remained rounded. The slender hair-like and coarser arm-like processes apparent in the others are fixed as seen during life. (On account of the continuous rapid change of shape, accurate camera-lucida drawings of such forms are not obtainable in life.) The flagellum is apparent in four of the figures.

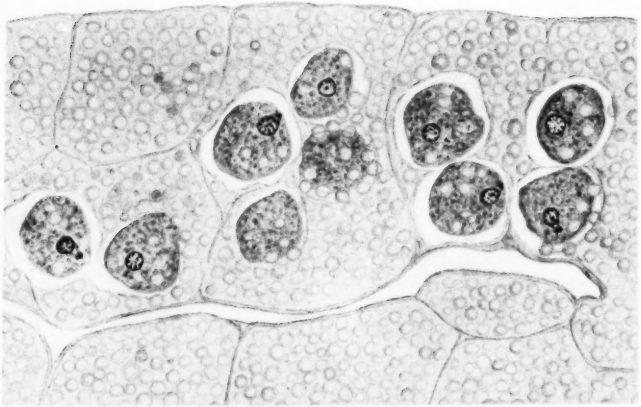
Fig. 41. Represents Histomonas assuming the form of a thin, clear sheet with irregular contour; sketched as accurately as possible from a living organism.

Fig. 44. Histomonas in extended form after Zenker fixation which caused the cytoplasm to appear granular throughout.

Fig. 47. Histomonas in the epithelium of the gut of an eleven-day old *Heterakis gallinae*. (The chicken from which material was derived showed acute blackhead, i. e., caecal involvement and liver lesions, the result of the experimental feeding of large numbers of Heterakis ova eleven days previously.) The protozoön developing in the intestinal epithelium of the worm has a dense granular cytoplasm, and apparently ingests the lipoid globules of the invaded cells. The blepharoplast is apparent in several of the organisms shown.

PLATE VI





47

Histomonas, fixed with pseudopodia extended (above); and in intestinal epithelium of caecal worm (below). X 1150.

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IV. LOSS OF VIRULENCE IN *HISTOMONAS MELEAGRIDIS* IN CULTURE AND THE IMMUNIZING PROPERTIES OF AN ATTENUATED STRAIN.

While *Histomonas meleagridis* has shown no appreciable modification in regard to its morphological characters in the course of long propagation in culture medium, biological changes as, for example, loss of virulence have become apparent. The records of experiments demonstrating such loss of virulence as well as the immunizing properties of an attenuated strain of this protozoön will be presented. In the development of Histomonas in its avian hosts, the protozoön may be found multiplying in the tissues, in which case it is associated with a disease process, or it may maintain itself indefinitely by multiplication in the caecal contents of apparently normal birds. Thus in the chicken, the disease blackhead, caused by the invasion of the tissues by Histomonas, is generally mild in character and of brief duration. The protozoön is eliminated from the tissues on recovery from the disease but usually continues to multiply in the caecal contents, thereby establishing the carrier state. Moreover, it is not improbable that in carriers the protozoön may from time to time invade the tissues to a slight extent, otherwise it is difficult to see how immunity is maintained. Thus we often have no means of ascertaining with certainty during life whether for any given period the protozoön is wholly excluded from the tissues or is now and again concerned in slight, transient invasions of the latter. In view of this uncertainty in regard to the exact distribution of the protozoön in carriers and also for the lack of another appropriate term, it is found convenient to refer to a case in which Histomonas has established itself, even though it may be confined to the caecal contents, as one of "infection." The term "superinfection" will also be employed in a similar sense implying the establishment of secondary strains of Histomonas in the caeca, irrespective of whether or not they invade the tissues.

Since the rectal injection of infective material has proved the most reliable and convenient method of producing a form of blackhead which agrees essentially with natural infections, it has been adopted as a routine procedure throughout the present investigation.

Strains of Histomonas and the Reactions of Inoculated Chickens and Turkeys.

The history of the first strain of Histomonas to become attenuated and which will be designated as Strain A, is briefly as follows:

On January 8, 1930, a laboratory-reared turkey was experimentally infected by furnishing it with food contaminated with henyard soil in which were large numbers of embryonated Heterakis eggs. This turkey developed blackhead and its liver lesions were ground in saline and the resulting suspension was rectally injected into a group of chickens. The infection that followed was transferred three days later to another group of chickens and from one of these Histomonas was isolated with associated bacteria in culture February 6, 1930. The isolation was made on the Locke-egg-serum medium of Boeck and Drbohlav (1925), but following the first few transfers on this medium, it has been propagated on the agar, saline, serum, starch medium described in a previous section. This strain being maintained in culture by weekly transplants was next employed to inoculate chickens on December 12 and 19, 1930, and on January 9, 1931. On each of these occasions all the inoculated birds showed extensive caecal disease and in some characteristic liver lesions also resulted from the inoculation. This strain of Histomonas had thus maintained its pathogenicity for a period of eleven months' growth in the culture tube. No further inoculations were made with Strain A until January 5, 1932, or twenty-three months after its isolation, when it was found to be almost completely innocuous for chickens. The inoculation of this attenuated strain serves to establish the carrier state at once, for it is possible to demonstrate Histomonas in the caecal discharges at almost any time following inoculation.

Substrain A 1 was isolated on June 21, 1932 from a chicken inoculated on March 19 of the same year with a culture of Strain A. Cultures of this Substrain have proved uniformly long lived and rich in Histomonas, but non-pathogenic.

The immunizing properties of an attenuated strain.—Since another newly isolated culture of Histomonas, Strain B, has been proved pathogenic for chickens, the protective properties of Strain A were next tested. Strain B was isolated in culture on February 27, 1932, from a fifty-eight day old laboratory-reared chicken that had been fed embryonated Heterakis eggs seven days previously. The results of the immunity-tests and control inoculations are presented in Table VII.

Stained sections of the minute opacities observed in the caecal wall in several of the vaccinated birds tested with Strain B showed foci in the mucosa infiltrated with Histomonas. Such minute lesions have been also occasionally encountered in infections from the Strain A culture and furthermore stained sections have revealed microscopic

TABLE VII.

THE PROTECTIVE EFFECT OF INFECTION WITH AN ATTENUATED STRAIN OF HISTOMONAS.

CHICK*	VACCINATION	TEST-INOCULATION	KILLED	POST MORTEM FINDINGS
8540	Jan. 20, 23, 28 and Feb. 6, rectally injected with Strain A cultures.	Mar. 5, rectally injected with Strain B cultures.	Mar. 11	Two minute opacities in caecal mucosa.
8541			Mar. 12	One minute opacity in caecal mucosa.
8542			Mar. 10	Normal
8543			Mar. 14	1.5 cm. opacity in caecal mucosa.
8544			Mar. 15	One minute opacity in caecal mucosa.
8545	None	Mar. 5, rectally injected with Strain B cultures.	Mar. 10	Caeca extensively diseased.
8546			Mar. 11	Caeca extensively diseased.
8547			Mar. 12	Caeca extensively diseased.
8548			Mar. 14	Caeca extensively diseased.
8549			Mar. 15	Caeca extensively diseased.
8550	None	Mar. 5, rectally injected with Strain A cultures.	Mar. 10	Normal, Histomonas present.
8551			Mar. 11	Normal, Histomonas present.
8552			Mar. 12	Normal, Histomonas present.
8553			Mar. 14	Normal, Histomonas not present.
8554			Mar. 15	Normal, Histomonas present.
8555	Mar. 5, rectally injected with Strain A cultures.	Mar. 15, rectally injected with Strain B cultures.	Mar. 21	Normal

* All chicks were Rhode Island Reds hatched on December 24 except 8555 hatched on January 26.

foci of infiltration with Histomonas in cases in which there was nothing abnormal to the naked eye in the appearance of the caecal mucosa. It is apparent from the experiment presented in Table VII that infection with the attenuated strain confers a high degree of protection against inoculations with a virulent strain of Histomonas.

That "superinfection" occurs as a result of the test-inoculation of immunized birds with virulent cultures is demonstrated by the pathogenicity of Histomonas derived from their caecal discharges. Thus after testing the immunity of Strain A carrier chickens by the rectal injection of the pathogenic Strain B Histomonas, the caecal discharges of six of these birds were collected five, seven, seven, eight, nine and ten days respectively after the test-inoculation and injected rectally into eight normal young chickens. These when killed after intervals of from six to eight days showed generalized caecal disease in six, focal inflammation in one, and no gross lesions at six days in the eighth bird, which, however, showed Histomonas in the caecal contents. Disease thus resulted from the inoculation of each of the six samples of caecal discharges obtained at intervals up to ten days after the inoculation of Strain A carriers with virulent Strain B Histomonas.

Successive passages of an attenuated strain through young birds.—During the course of these experiments, attempts were made to restore the virulence of the attenuated Strain A by rapid passages through both chickens and turkeys, but without success. Thus passage through five lots of chickens inoculated in succession and later on through five successively inoculated young turkeys during a period of seven and a half weeks has failed to produce disease. Neither has this strain become pathogenic through a sojourn of several months in a chicken carrier. On account of its constancy with reference to its non-pathogenicity, the attenuated strain to some extent resembles a "*virus fixé*."

Possible effects of associated organisms on virulence.—The question of whether the difference in respect to pathogenicity now apparent in the two strains is based on the character of the organisms associated with Histomonas in the culture tube, has been considered. With the view of separating the associated organisms from Histomonas in the virulent culture, the protozoön was killed by freezing cultures overnight at $-15°$ C. The effects of the inoculations of chickens either with the bacterial content of virulent Strain B cultures alone, or in mixture with the attenuated Strain A cultures were next determined. The essential data are furnished in Table VIII. As shown by the results here tabulated, the bacterial content of Strain B culture does

TABLE VIII.

THE EFFECT OF ORGANISMS ASSOCIATED WITH HISTOMONAS IN CULTURE ON PATHOGENICITY.

CHICK*	INOCULATION	HISTOMONAS	DATE KILLED	POST MORTEM FINDINGS
8632	May 17, rectally injected with Strain A cultures of Histomonas.	Present	May 23	Normal
8633		"	May 24	Normal
8634		"	May 25	Normal
8635		"	May 26	Minute opacity in caecal mucosa.
8636		"	May 27	Normal
8637		"	May 28	Minute opacities in caecal mucosa.
8638	May 17, rectally injected with a mixture of Strain A cultures and frozen Strain B cultures.	Present	May 23	Normal
8639		"	May 24	Normal
8640		"	May 25	Normal
8641		"	May 26	Normal
8642		"	May 27	Normal
8643	May 17, rectally injected with frozen Strain B cultures.	Absent	May 23	Normal
8644		"	May 24	Normal
8645		"	May 25	Normal
8646		"	May 26	Normal
8647		"	May 27	Normal
8648	May 17, rectally injected with Strain B cultures.	Present	May 25	Liver and caeca extensively diseased.
8649		"	May 23	Liver and caeca extensively diseased.
8650		"	May 26	Liver and caeca extensively diseased.
8651		"	May 27	Small spots in liver, caeca extensively diseased.
8652		"	May 24	Liver and caeca extensively diseased.
8653	May 17, rectally injected with a mixture of Strain A and Strain B cultures	Present	May 25	Caeca extensively diseased.
8654		"	May 27	Liver and caeca extensively diseased.
8655		"	May 23	Liver and caeca extensively diseased.
8656		"	May 23	Liver and caeca extensively diseased.
8657		"	May 26	Caeca extensively diseased.

* All chicks were Rhode Island Reds hatched on May 10.

not appear to be a significant factor in the pathogenicity of this strain. Results identical with those here presented had been previously obtained in another similar experiment in which Histomonas was eliminated from Strain B cultures by freezing the latter for brief periods with longer intervals at room temperature. In that experiment, a series of twenty chicks were employed, being divided into four groups of five each and inoculated respectively with Strain B, Strain A, a mixture of Strain A and frozen Strain B, and with frozen Strain B.

Vaccination of young turkeys.—Having found that infection with the attenuated Strain A Histomonas serves to protect chickens against blackhead, protective inoculations were then attempted in young turkeys. Incubator-hatched turkey poults of four different lots were vaccinated at an early age by the rectal inoculation of a mixture of Strain A and Substrain A 1 Histomonas.

These lots, designated numerically, were as follows: Lot I was comprised of twenty poults, eight hatched on May 3 and twelve on May 8; Lot II, twenty-seven poults hatched June 5; Lot III, thirty-three poults hatched June 30; and Lot IV, nine hatched August 3, 1932. Lots I and III were obtained directly from the incubator. Lot II consisted of birds from a poor hatch following a great elevation of incubator-temperature toward the end of incubation, and these birds were also exposed to brooder house conditions for two days after removal from the incubator. Lot IV had been in the brooder house for twenty-four hours after removal from the incubator, before being taken to the laboratory.

Not included in Lot III were two poults of the same hatch that died at the age of seven days, one of which showed caecal cores but no evidence of blackhead in study of stained sections, and another killed by accident at the age of fifteen days. Not included in Lot IV were three poults which died in the first week of life, two showing the non-specific typhlitis with caecal cores described in Section II.

Sixteen turkeys of Lot I were vaccinated with Strain A Histomonas cultures and from seventeen to nineteen days later were inoculated with Strain B cultures as an immunity-test. Following the test-inoculation, one of these succumbed to caecal histomoniasis while all four similarly inoculated control turkeys died with extensive liver and caecal involvement (see Table IX).

The vaccinated turkeys of Lot II were not given immunity-test inoculations until three months after vaccination, but were in the meantime exposed to contaminated soil. Seventeen turkeys of this lot developed caecal induration in the first two or three weeks of life, this condition appearing before inoculation in six of a group of nine. One that died five days after inoculation showed caecal cores but no

evidence of blackhead, and seven others that either died or were killed showed slight invasion of the tissues by Histomonas, in two there were also several minute spots in the liver, but in none of these infected birds was the infiltration with Histomonas extensive. In two, death was obviously due to intercurrent infection, there being pericarditis in one and peritonitis in another. The demonstration of two cases of histomoniasis in two uninoculated poults further complicates the results obtained in this experiment, and might be regarded as evidence of maternal transmission of infection through the egg. This conclusion is not, however, justified, on account of a period of exposure of all of the birds of Lot II to brooder house conditions.

Lot II has thus furnished results of little value apart from the fact that it furnishes a group of turkeys exposed early in life to a non-virulent Histomonas infection. The immediate effects of the vaccination cannot be judged on account of the intercurrent conditions, i. e. nonspecific typhlitis and bacterial infections. Data pertaining to this lot are summarized in Table X.

In Lot III, twenty-nine poults were vaccinated and four of these when subjected several weeks later to immunity-test inoculations,—two being fed Heterakis ova and two being injected with virulent culture,—were shown to be adequately protected while the four similarly inoculated control turkeys succumbed to typical blackhead (see Table IX). The vaccination of the twenty-nine poults was followed by three deaths and in each of these cases there was invasion of the caecal mucosa by Histomonas, clearly accounting for the death in one, but in the other two which showed visceral anomalies (see Table X), the slight degree of histomoniasis probably had little if any effect on the health of the bird.

The vaccination of seven poults of Lot IV resulted in the protection of all seven to subsequent inoculations of virulent culture (see Table IX). One of the two controls inoculated with the latter, died of typical blackhead, the other passed through a long period of illness and recovered, but was of no commercial value. When killed two months later, the liver was extensively scarred and lobulated throughout. This turkey was the only one that survived in a total of ten controls that received test-inoculations with virulent Histomonas.

All four lots of turkeys were exposed subsequent to their vaccination to farm conditions which had in previous years never failed to produce blackhead. Since several cases of clinical blackhead eventually appeared in the second (June 5th) lot, the birds of this and the third (June 30th) lot were given a test-inoculation with virulent cultures late in the season, that is on October first and October eleventh.

Although there had been two fatal and three non-fatal cases of blackhead in the second (June 5th) lot of vaccinated turkeys, the late test-inoculation furnished no additional cases. In the third

TABLE IX.

BLACKHEAD VACCINATION AND IMMUNITY TEST-INOCULATIONS IN TURKEYS.

	TURKEY	HATCHED	VACCINATION	RESULT	TEST-INOCULATIONS	RESULT
Lot I	8628	May 3	May 10	No symptoms	May 27, June 4 & 6	No symptoms. Killed July 31, caecum scarred.
	8629	" "	May 16	No symptoms	June 4 & 6	No symptoms
	8630	" "	" "	No symptoms	" " "	No symptoms
	8631	" "	" "	No symptoms	" " "	No symptoms
	8658	" "	May 20	No symptoms	June 6 & 8	No symptoms
	8659	" "	May 20 & 24	No symptoms	" " "	No symptoms
	8660	" "	May 20	No symptoms	" " "	No symptoms
	8661	" "	" "	No symptoms	" " "	Caecal induration June 18. Recovered.
	8662	May 8	" "	No symptoms	" " "	Died June 23. Caecal blackhead.
	8663	" "	" "	No symptoms	" " "	No symptoms
	8664	" "	" "	No symptoms	" " "	No symptoms
	8665	" "	" "	No symptoms	" " "	No symptoms
	8666	" "	" "	No symptoms	" " "	No symptoms
	8667	" "	" "	No symptoms	" " "	No symptoms
	8668	" "	" "	No symptoms	" " "	No symptoms
	8669	" "	" "	No symptoms	" " "	No symptoms. Killed June 20, caeca normal.
	8670	May 8	None (Control)	.....	June 4 & 6	Died June 16. Typical blackhead.
	8671	" "	" "	.....	June 6 & 8	Died June 15. Typical blackhead.
	8672	" "	" "	.....	" " "	Died June 17. Typical blackhead.
	8673	" "	" "	.....	May 27 & June 4	Died June 7. Typical blackhead.

TABLE IX—(*Continued*).

BLACKHEAD VACCINATION AND IMMUNITY TEST-INOCULATIONS IN TURKEYS.

	TURKEY	HATCHED	VACCINATION	RESULT	TEST-INOCULATIONS	RESULT
From Lot III	8705	June 30	July 2 & 15	Caecal induration	August 2	No symptoms. Killed August 30, normal.
	8721	" "	July 6, 14 & 15	No symptoms	July 27 & 29	No symptoms
	8702	" "	July 2	No symptoms	July 27*	No symptoms
	8704	" "	" "	No symptoms	July 27*	No symptoms
	8748	June 30	None (Control)	.....	July 27*	Dead August 11. Typical blackhead.
	8749	" "	" "	.....	July 27*	Dead August 15. Typical blackhead.
	8750	" "	" "	.....	July 27 & 29	Dead August 8. Typical blackhead.
	8751	" "	" "	.....	July 27	Dead August 5. Typical blackhead.
Lot IV	8765	Aug. 3	August 6	Caecal induration	October 1 & 11	No symptoms
	8766	" "	August 15 & 17	No symptoms	" " "	No symptoms
	8770	" "	August 20	Caecal induration	" " "	No symptoms
	8771	" "	Sept. 2	No symptoms	" " "	No symptoms
	8772	" "	" "	No symptoms	" " "	Slight induration Oct. 11. Recovered.
	8773	" "	Sept. 12	No symptoms	" " "	No symptoms
	8774	" "	Sept. 27	No symptoms	Oct. 11	No symptoms
	8775	Aug. 3	None (Control)	.....	Oct. 1	Symptoms Oct. 11. Recovered. Killed Dec. 2. Liver scarred.
	8776	" "	" "	.....	" "	Died Oct. 16. Typical blackhead.

* Fed embryonated Heterakis ova.

TABLE X.

VACCINATION FOLLOWED BY EXPOSURE TO CONTAMINATED SOIL AND LATE TEST-INOCULATIONS.

	VACCINATED TURKEYS	POST-VACCINAL DISEASE	IMMUNITY TESTS	RESULT
Lot II*	Twenty-seven poults hatched June 5. June 8, eighteen were rectally injected with sediment of cultures of Strain A. Two of these and three of the other nine turkeys having shown no caecal induration were vaccinated by rectal injection on June 30.	June 13, one died showing caecal cores but no Histomonas in the tissues. Seventeen, including six of the nine uninoculated poults, developed induration of the caeca. Two that died and five that were killed during June showed invasion of the caecal mucosa by Histomonas. Several small liver lesions were found in one of the uninoculated poults.	June 30, the nineteen survivors were placed on contaminated soil.	Aug. 23, one died, typical blackhead. Sept. 14, one died, typical blackhead. Up to Oct. 11, three other non-fatal cases of blackhead appeared.
			Oct. 11, fifteen of the seventeen survivors (two recently recovered from blackhead not included) were injected rectally with virulent cultures of Histomonas, Strains B and D.	No case of blackhead subsequent to the test-inoculation.
From Lot III	Twenty-five poults hatched June 30. Vaccinated with attenuated Strain A Histomonas in successive lots on various dates from July 3 to July 15 and reinjected on subsequent dates as necessary until infection was established as shown by microscopic examination of discharges.	July 15, one poult with a large diverticulum of gizzard was killed, showing no gross lesions of blackhead but microscopic invasion of the caecal mucosa. July 26, a runt with large yolk sac died showing peritonitis and caecal blackhead. Aug. 19, a poult died of progressive caecal blackhead of more than 3 weeks' duration. Another bird showing caecal induration recovered later on.	During last week in July, the twenty-two survivors were placed on contaminated soil.	Sept. 30, one bird showed symptoms of blackhead but had recovered five days later.
			Oct. 1, all twenty-two survivors and on Oct. 11 all those not showing symptoms received rectal injections of virulent cultures of Histomonas, Strains B and D.	Between Oct. 7 and Oct. 21, ten cases of clinical blackhead appeared. All birds recovered although one was critically ill for several days. No sign of disease has subsequently appeared.

*That the poults of this group were from a poor hatch resulting from exposure to a period of high incubator temperature and were also exposed for two days to brooder house conditions, may account for their condition following vaccination.

(June 30th) lot, the test-inoculations resulted in ten cases of clinical blackhead, but every affected bird recovered.

Strain D, a more recently isolated strain of Histomonas, was employed in the late immunity test-inoculations. This was obtained on August 3, 1932 from the caecal discharges of a thirty-four day old turkey fed embryonated Heterakis eggs seven days previously.

The results of the vaccination of the above four lots of turkeys may be summarized as follows. In poults of Lot II, some of which had shown at an early age occasional cases of nonspecific caecal obstruction, and which had also been exposed for a time to brooder house environment, the inoculation of Strain A Histomonas has been attended with a high incidence of caecal disease. Metastasis to the liver, however, has rarely occurred as far as may be judged from fatal cases in these groups, and the caecal infection was also in general nonprogressive. The morbidity observed early in life in this group of birds is obviously not attributable to the vaccination alone, for the histological study of the tissues shows a much more intense inflammatory reaction than would be associated with so mild a degree of histomoniasis, if uncomplicated. The occurrence of pericarditis and peritonitis in the fatal cases also furnishes evidence of infection other than histomoniasis. If, however, Lot II is included in our figures on mortality due to vaccination and assuming that the poults that were killed would have otherwise died, there were eight deaths in the seventy-eight turkey poults thus far vaccinated (omitting one that died at the age of five days), or approximately ten per cent mortality. If Lot II is omitted on account of factors other than vaccination contributing to mortality, there was one death in fifty-two young turkeys vaccinated, or less than two per cent mortality from vaccination. Either of these figures is in marked contrast to nine deaths in ten turkeys, or 90 per cent mortality following inoculation with virulent strains of Histomonas, and the tenth turkey, while surviving the disease was permanently damaged to such an extent as to render it worthless.

The immunity of vaccinated young turkeys has been tested by the inoculation of virulent strains of Histomonas at various intervals up to eight weeks after vaccination, Table IX. There was one death in twenty-seven poults thus tested, or a mortality of 3.7 per cent attributable to the test inoculation, and in this case although there was progressive caecal disease, there was no liver involvement. Again these results may be compared with 100 per cent liver involvement and

90 per cent mortality in the control turkeys of the same lots inoculated on the same occasions with identical material.

That the protection conferred by the attenuated strain may be lost in the course of time is shown by the appearance of several cases of blackhead in turkeys two or more months after vaccination. Also when the immunity test-inoculations are postponed until several months after vaccination, partial loss of protection is indicated in the appearance within a fortnight after the test inoculation of cases of clinical but non-fatal blackhead. While it is probable that successive transient infections tend to build up immunity, it is believed, although not actually demonstrated in these experiments, that loss of immunity is preceded by loss of infection,—that is a discontinuance of the carrier state. That the infection may be lost is shown by the records of two cases of histomoniasis in laboratory-reared turkeys, in which Histomonas disappeared after a time from the caecal discharges, being no longer demonstrable by direct examination or by culture, and the caecal material proving noninfective when inoculated into normal birds. In histomoniasis, immunity is probably maintained at a high level on continuous exposure to infection, such as may be derived from soil sown with Heterakis eggs or from association with other infected birds of the flock.

The reinforcement of vaccination by test-inoculations of a virulent strain of Histomonas has resulted in an effective immunity which has withstood exposure to contaminated soil throughout the summer and autumn. An index of the contamination of the soil is furnished by the high incidence of the caecal worm, *Heterakis gallinae*, in experimental turkeys when prepared for market late in November,—1034 worms were obtained from the caeca of a single bird. In order to determine whether Histomonas had persisted in the flock up to this time, the embryonated eggs from Heterakis collected from a number of these turkeys were fed to young chickens. Blackhead followed the feeding of five out of eleven samples of Heterakis material from individual turkeys, proving that at least a large proportion of these apparently normal turkeys had remained carriers of virulent blackhead.

Attempted vaccination of mature turkeys.—Since a considerable mortality from blackhead is frequently observed in year-old turkey hens toward the end of the laying season, a group comprised of three males and sixteen females from the lots vaccinated early in life has been held over to the present season (1933). Opportunity was also taken to compare the effects on mature turkeys of vaccination against blackhead early in life with that attempted at maturity. Thus sixteen

turkey pullets were purchased on March 8, 1933, from a flock considered to be free from blackhead. The turkey raiser from whom these were obtained reared from ten to twelve thousand turkeys in the summer of 1932 and reported that he had observed no blackhead. A casual inspection of his flock on several occasions had revealed no birds with suggestive symptoms. Nevertheless no certain information is available as to the possible presence of avirulent Histomonas infection, although in view of the great numbers of birds congregated together without evidence of disease, this does not seem probable. Eight of these purchased turkeys were vaccinated with the attenuated Strain A Histomonas on March 9 and March 11, 1933. On account of the difficulty in obtaining caecal discharges from individual mature birds, and also in the recognition of Histomonas in the presence of swarms of other species of protozoa, no attempt was made to determine whether this organism became established in these birds. About a fortnight later, the eight vaccinated and the eight non-vaccinated control turkeys were added to the flock of nineteen birds vaccinated early in life and exposed henceforth to contaminated soil, including a tract spread thickly with poultry manure.

At the time of writing, July 31, 1933, there has been one death from undetermined causes from the nineteen breeding turkeys vaccinated early in life. Six of the eight vaccinated in March 1933 have died, the deaths of three of these being directly attributable to blackhead, and the scars of this disease contributing to the deaths of the other three. Three of the eight non-vaccinated controls have died, two from blackhead and one from other causes.

Thus the vaccination of turkeys at maturity has furnished no adequate protection while vaccination early in life with more or less continuous exposure thereafter to contaminated soil, has completely protected a flock of nineteen turkeys. Incidentally it is of interest that a considerable proportion of non-vaccinated adult turkeys survive for relatively long periods under conditions that would be promptly fatal to young turkeys. An explanation of this failure of the inoculation of Strain A Histomonas to protect mature turkeys is furnished by the results obtained in experiments carried out several months later on young chickens and turkeys.

Loss of immunizing properties under long cultivation.—Since it was not known to what extent Strain A had retained its immunizing properties during its third year under cultivation, a group of seven chickens inoculated on May 26, 1933, with this strain were subjected on June 24 to a test-inoculation with virulent Strain D isolated some-

what over ten months previously. All but one of these vaccinated chickens showed some degree of disease when killed six days later, but slight in contrast with that found in a series of eight non-vaccinated control chickens of the same hatch simultaneously inoculated with the same virulent strain and killed on the same date. The lesions of the most severely affected chicken of the vaccinated series were less extensive than those of the mildest case of the control non-vaccinated series. Thus while the caeca of all but one of the vaccinated chickens presented macroscopic lesions, they were nevertheless contractile and functioning. Those of the non-vaccinated chickens, which were diseased throughout, showed rigid non-contractile walls and in all except one bird were distended with large cores of exudate. Since no lesions of consequence were found the year previously in vaccinated chickens tested with virulent culture, it is clear that Strain A has lost some of its immunizing properties under continued cultivation. This may account for the failure of this strain to protect the mature turkeys vaccinated several months previously.

Virulence tests of different strains of Histomonas on day-old chickens.—In order to test the pathogenicity of the two strains of Histomonas isolated in culture during the year 1932, two groups of day-old chickens have been inoculated, one with Strain B of March 16, 1932, the other with Strain D of August 3, 1932. In each group, one bird was killed five days, one six days, and four seven days after inoculation. Histomonas became established in all these birds, but those inoculated with Strain B showed no lesions while those inoculated with Strain D showed extensive caecal disease and also minute liver lesions in three instances. At the same time, two groups of chickens were inoculated, one with Strain A and one with Substrain A 1, previously shown by earlier experiments to have become attenuated. In each group, three birds were killed at the end of seven days, one eight days, and one ten days after inoculation. With the exception of small opacities in one bird inoculated with Substrain A 1, none of these showed lesions, although Histomonas was established in all. Thus not only has the older Strain A with its Substrain A 1 continued to be non-pathogenic, but Strain B has apparently lost pathogenicity for chickens. Attention is called to the fact that a year previous to this, Strain B produced blackhead with liver involvement with great regularity.

Vaccination of young turkeys in 1933.—A commercial flock of fifty young turkey poults were inoculated with Strain A on May 31 of the present year, and during the three weeks following showed no manifestation of disease. Those in which Histomonas was not demon-

strated were reinoculated on June 6 and some again on June 10. On June 24, all fifty were rectally injected with cultures of Strain B which, as shown above, has lost much of its pathogenicity for chickens. From ten to fourteen days later, as many as eight of these birds showed loss of appetite, inactivity, and in most of these the caeca were found on palpation to be distinctly indurated. One poult died on July 14 and one on July 18, both from blackhead with a moderate number of liver lesions. One dying on July 25 showed focal caecal lesions and peritonitis. Two died with no indication of blackhead, and forty-five survive. The birds had promptly acquired considerable numbers of Heterakis as well as Ascaridia from their exposure to soil since June 9. The vaccination of this flock of turkeys was done for practical rather than for scientific purposes, and should not be regarded as a properly conducted experiment. The occurrence of typical cases of blackhead may be explained either through the acquisition of inadequately protected birds of a virulent strain of Histomonas from exposure to soil, or by the possibility that Strain B, while innocuous for chickens, is still pathogenic for turkeys. Hence additional inoculations were made in chickens and in young turkeys in order to test further the pathogenicity of Strain B.

Loss of virulence in a second strain of Histomonas.—Five chickens were injected rectally with Strain B cultures and three of these were killed after nine days, and two, eleven days later. One of the five showed foci of thickening of the caecal mucosa, but the caeca of the other four appeared normal. Young turkeys were also inoculated on the same occasion with Strain B and, with the exception of one which failed to become infected, all developed a mild form of histomoniasis with focal or generalized thickening of the caecal mucosa, but without ulceration or involvement of the liver. None of these showed symptoms and all grew normally up to the time that they were killed. Cores of exudate were present in several but were non-adherent. The disease was obviously nonprogressive in these turkeys and in several the process of repair was well advanced when the bird was killed. Data pertaining to the virulence tests of Strain B are furnished in Table XI.

It is evident from these inoculations that Strain B after sixteen months in culture now rarely produces lesions in chickens and is only slightly pathogenic for young turkeys. In the latter, it produces a mild nonprogressive disease of the caeca and has now lost most of its metastasizing qualities. Hence it seems probable that a progressive loss of virulence may take place with considerable regularity in Histomonas propagated in culture medium.

TABLE XI.

VIRULENCE TEST-INOCULATIONS OF STRAIN B AND STRAIN D HISTOMONAS, AFTER LONG CULTIVATION.

BIRD	AGE	INOCULATION	RESULT
C 8858	2 days	May 26, 1933, rectally injected with culture of Strain B Histomonas isolated March 16, 1932.	K. May 31. Normal, Histomonas present.
C 8859	" "		K. June 1. Normal, Histomonas present.
C 8860	" "		K. June 2. Normal, Histomonas present.
C 8861	" "		K. June 2. Normal, Histomonas present.
C 8862	" "		K. June 2. Normal, Histomonas present.
C 8863	" "		K. June 2. Normal, Histomonas present.
C 8864	2 days	May 26, 1933, rectally injected with culture of Strain D Histomonas isolated August 3, 1932.	K. May 31. Caeca extensively diseased.
C 8865	" "		K. June 1. Caeca extensively diseased.
C 8866	" "		K. June 2. Caeca extensively diseased, one liver lesion.
C 8867	" "		K. June 2. Caeca extensively diseased, minute liver lesions.
C 8868	" "		K. June 2. Caeca extensively diseased, two minute liver lesions.
C 8869	" "		K. June 2. Scattered and confluent focal lesions of caeca.
T 8928	6 days	July 17, 1933, rectally injected with culture of Strain B Histomonas isolated March 16, 1932.	K. July 26. Mild caecal disease* with core.
T 8929	" "		K. July 26. No infection.
T 8930	" "		K. July 26. Caecal lesions 3–8 mm.
T 8931	" "		K. July 28. Mild caecal disease with core.
T 8932	" "		K. July 28. Mild caecal disease, healing.
T 8933	" "		K. July 29. Small foci of infection in caeca.
T 8934	" "		K. July 29. Focal lesions of caeca, slightly adherent masses of exudate.
T 8935	" "		K. Aug. 7. No lesions, Histomonas present.
T 8936	8 weeks	Same	K. July 28. Extensive non-ulcerative caecal disease with cores.
C 8937	27 days	Same	K. July 26. Mild caecal disease.
C 8938	" "		K. July 26. Normal, Histomonas present.
C 8939	" "		K. July 28. Normal, Histomonas present.
C 8940	" "		K. July 28. Normal, Histomonas present.
C 8941	2 months	Same	K. July 26. Normal, Histomonas present.

* Mild caecal disease signifies either small focal lesions or thickening of caecal wall without ulceration.

Discussion

Histomonas meleagridis in the course of long cultivation in association with the bacteria with which it is isolated apparently tends to lose virulence. Thus a strain (Strain A) of the organism, which for a time produced liver lesions as well as caecal disease in young chickens, has become non-pathogenic for both chickens and turkeys. A second strain (Strain B) of the protozoön which has been under cultivation for sixteen months, and also originally fully virulent, now rarely produces lesions in chickens and is only slightly pathogenic for young turkeys. Infection with a strain which has become non-pathogenic under cultivation has served to protect chickens and turkeys against blackhead, but after an additional year under cultivation, its immunizing properties appear to have diminished.

Virulent Histomonas may be readily obtained in culture from chicken carriers produced either by the feeding of Histomonas-bearing Heterakis eggs or by the rectal injection of liver lesions from acute cases of blackhead. By isolating such cultures at long intervals, as, for example, yearly, it is probable that strains may be made available that will show progressive loss of virulence and immunizing properties. The partial protection afforded by non-pathogenic strains may be reinforced by superinfection with pathogenic strains.

The advantages to be derived from the vaccination of turkeys early in life against blackhead are obvious. There is not only the factor of economy in the saving of labor, reduced outlay for feeds and for wire platforms and other equipment, but it is possible to afford conditions which are in the long run favorable to the development of a vigorous stock. Thus by rearing young turkeys on ground from early life, they exercise freely, are able to dust themselves, obtain more or less in the way of vegetable and mineral matter, and a certain number of insects. Occupation in foraging for green stuff and insects maintains a morale in a flock not often observed in platform-reared birds. Even though small losses may attend the procedure of vaccination, such are to be preferred to the loss of similar numbers later in life. However, while adequate protection may be afforded when vaccination is carried out under laboratory conditions, as shown by the results of the present investigation, unfortunately there are serious obstacles to employing vaccination in a commercial way. Thus difficulty is encountered at once in the matter of distributing attenuated cultures, for Histomonas will not survive in culture at ordinary room temperatures. While there are means of overcoming

this difficulty, there is the element of uncertainty in producing infection even when each bird is carefully injected with Histomonas culture. The inoculation of turkeys is apparently less uniformly successful than the inoculation of chickens. In order to produce an effective immunity, the vaccination with an attenuated culture should be followed in several weeks by the inoculation of a virulent culture. The gradual loss of the immunizing properties of the first attenuated strain studied also indicates that continued isolations at intervals of strains of Histomonas would be necessary in order to have cultures on hand that are non-pathogenic but nevertheless possess immunizing properties. Furthermore, even an attenuated strain may be regarded as potentially harmful to poults of low vitality or those that are in any way abnormal. Unless these obstacles are in some way overcome, the vaccination of blackhead may not be expected to be placed on a practical basis.

The prompt development of an effective protective reaction as the result of infection with an attenuated strain of Histomonas was quite unexpected and appears to be an unusual phenomenon in protozoan infection. However, this immunity has certain features in common with that found in many other protozoan infections in that it apparently requires the presence of the protozoön, that is the carrier state, for its maintenance and tends to disappear with the loss of infection. Further studies are essential in order to determine the nature of this immunity, but such observations as have been made indicate that it is based on a change in respect to the local reaction of the host tissues to the invading protozoön.

The hypothesis of Graybill and Smith (1920) that the development in the caeca of large numbers of Heterakis may lower the birds' resistance and so favor the invasion of the tissues by the protozoön may be reconsidered in connection with the exposure of the carriers produced by vaccination, to heavy infestations of the worm. The study of material derived from a considerable number of chickens first vaccinated by the rectal injection of cultures of attenuated Histomonas and later fed Heterakis eggs, has shown that the developing worms may produce punctate hemorrhage and exudate, in some instances in sufficient amount to fill the blind end of the caeca, but in no such instance has any invasion of the tissue of the immunized bird by Histomonas been found. Two vaccinated, young turkeys, see Table IX, 8702 and 8704, showed no symptoms,—one developed transient induration in the region of the caeca subsequent to the feeding of large numbers of Heterakis eggs, while two similarly fed

controls died of blackhead. Thus while there is ample evidence that the development of Heterakis is productive of injury to the caecal mucosa, and also that the protozoön causing blackhead may be introduced with the eggs of the worm, nevertheless the presence of large numbers of the developing worms has failed to produce acute blackhead in the carrier.

We have no definite information as to the extent to which the pathogenicity of Histomonas varies in nature. Certain observations made in the course of previous investigations indicate that relatively mild strains may occur. Thus it was found in our earlier investigations (Tyzzer and Fabyan, 1922) that the inoculation of the breast of the turkey with material from liver lesions of acute blackhead produced a progressive and uniformly fatal disease. Later on in a series of similar inoculations with liver lesions from another case, a considerable proportion of the breast lesions regressed and the birds fully recovered. The occurrence of mild strains of Histomonas in nature would also account for the occasional instances of the successful rearing of turkeys under surroundings that would appear obviously unfavorable.

Summary

A strain of *Histomonas meleagridis* propagated in nutrient media in association with bacteria was demonstrated to be virulent after eleven months in culture but had lost its pathogenicity at the end of twenty-three months.

The immunizing properties of this strain against a recently isolated, fully virulent strain were demonstrated in the spring and summer of 1932 by experiments on young chickens and turkeys kept under rigidly controlled experimental conditions as well as by the practical test of rearing a vaccinated flock of turkeys for market under conditions in which non-vaccinated turkeys would not be expected to survive. This strain after another year in culture now only partially protects chickens against a virulent strain of Histomonas.

The inoculation of carriers, infected with an attenuated strain of Histomonas, with a virulent strain commonly results in the establishment of the latter in the caeca, or "superinfection."

The inoculation of the carrier with virulent strains of Histomonas not only probably reinforces its immunity but also furnishes a basis for the continued exposure to virulent infection necessary for subsequent freedom from disease.

Rapid passages of an attenuated strain of Histomonas through young chickens and turkeys have failed to restore its virulence.

The virulence of a Histomonas culture is found not to be dependent on the nature of the bacteria occurring in association with the protozoön but is a manifestation of the biological character of the latter, and is a quality which may be modified by long continued propagation in vitro.

A second strain of Histomonas isolated on February 27, 1932 and remaining fully virulent during the following summer, has lost much of its pathogenicity for young turkeys after sixteen months in culture and is almost wholly non-pathogenic for chickens. The third strain isolated August 3, 1932 was found to be fully virulent nearly ten months later.

On the basis of histological evidence, it appears probable that the immunity resulting from the inoculation of an attenuated strain of Histomonas is due to slight and non-progressive invasions of the tissues by the protozoön. Thus while macroscopic lesions seldom appear, the study of stained sections reveals Histomonas in such minute opacities as occasionally occur and in some instances in caecal mucosa that appears quite normal to the naked eye.

The suggestion is made that diminution in the immunizing properties of Histomonas under continued cultivation may be attendant upon a further loss of ability to invade the tissues. In the course of long cultivation, Histomonas appears not to have lost infectivity, i. e. ability to establish itself in the caeca of chickens and turkeys. No evidence has been obtained thus far that loss of virulence in Histomonas is attended with greater chronicity of disease, for young turkeys recover promptly from infections with a strain formerly virulent but at present only mildly pathogenic.

It is pointed out that serious obstacles interfere with the placing of the vaccination of turkeys on a practical basis.

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